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THE BIOASSAY OF GLUCO-CORTICOID ACTIVITY BY THE MOUSE GLYCOGEN METHOD: SOME NEW FACTS

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EXPERIENCE with the mouse glycogen method for the bioassay of gluco-corticoid activity was obtained while investigating the conversion of steroids by adrenal tissue. A number of new facts emerged, some of which of primary importance in the assessment of the accuracy and merit of different bioassay procedures. The method of Venning *et al.* (1946) measures the deposition of glycogen in the depleted livers of fasted adrenalectomized mice, whereas the methods of Eggleston *et al.* (1946) and of Dorfman *et al.* (1946) depend on maintaining the well-stocked depots of hepatic glycogen during fasting. Venning's method appeared to possess greater accuracy, and was adopted for the present investigation.

METHODS

Operative technique and anaesthesia. The adrenals were removed by the lumbar approach, at first under pentobarbitone-ether anaesthesia, but better results were obtained with combined local and intraperitoneal xylocaine. The intraperitoneal dose produced anaesthesia of the peritoneum, as well as a state of stupor during which the operation was carried out. Mice weighing 20–26 g. received 0.15 ml. of 1% xylocaine without adrenaline subcutaneously and 0.15 ml. intraperitoneally. For mice under 20 gm., the intraperitoneal dose was 0.1 ml. Half the subcutaneous dose was placed over the right lumbar peritoneal incision and half over the left, and the operation performed without pain response 5–10 minutes after the injection of xylocaine. The tail of the mouse was fixed by adhesive plaster, and its right ear held by an assistant. The animals suffered less discomfort and shock than with ether. By the fourth postoperative day, the mortality for 250 mice adrenalectomized under pentobarbitone and ether was 20%, while the mortality for 1682 mice adrenalectomised under xylocaine was 6.9%.

Strain of mice. Venning *et al.* used pure strain mice from their "own colony" but in the present work reliance had to be placed on dealers for the uniformity of the strain of mice.

The "constant temperature room." Venning *et al.* kept their adrenalectomized animals in a "constant temperature room" at 76° F. In the present work, the temperature was

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kept above 70° F. by a thermostat, but there were no means of reducing high temperatures in the summer. The temperature of the animal room was recorded three times daily, however, and a study made of the effects of its variation.

RESULTS

Preliminary assays on adrenal cortical extract, *Eucortone*, showed marked individual variation. This variation was particularly noticeable in the 20 control animals, four of which gave values over 10 mg. glycogen per 100 gm. body weight, *viz.*, 26.3, 28.5, 66.7 and 67.2. The mean and its standard error were 10.86 ± 4.64, while the figures of Venning *et al.* (34 controls) were 4.1 ± 0.5, showing a marked difference in the mean, and a more striking difference in its standard error. Postmortem failed to reveal remnants of glands or accessory adrenal rests. The causes of this high variation were next investigated.

Liver glycogen levels in normal and in fasted non-adrenalectomized mice. The standard error of the mean liver glycogen (Table 1) reveals again wide

TABLE 1. LIVER GLYCOGEN LEVELS IN NORMAL NON-FASTED MICE, AND IN NORMAL MICE FASTED FOR DIFFERENT PERIODS

Group	Number of animals	Period of fast in hours	Liver glycogen per 100 gm. mouse mean ±S.E.	Coefficient of variation
a	30	0	81.08 ± 11.53	77.9
b	10	8	40.62 ± 10.12	78.8
c	30	24	16.80 ± 3.7	120.5
d	20	24	24.35 ± 5.91	108.5

variation in all four groups, a variation which in this case could not be ascribed to faulty operative technique.

The coefficient of variation was found useful, particularly for comparing the present results with those of other workers, and led to interesting conclusions. In biological measurements, coefficients greatly exceeding 25% indicate a multiplicity of variables and necessitate the use of large numbers of animals in assays. A study of the factors concerned with the object of reducing the coefficient of variation is then desirable, and may result in a considerable increase in the accuracy of such assays.

The coefficient of variation of the first 20 control adrenalectomized mice was 191.2, while the coefficients for normal and fasted normal mice are shown in Table 1. An inverse proportionality is noted between the mean and its coefficient of variation in the latter group, so that the higher coefficient of the adrenalectomized controls could be attributed to a lower mean. A later group of starved *normal* mice gave a mean liver glycogen of 8.4 and a variation coefficient of 218.56. The technique of adrenalectomy was evidently exonerated.

The precision of the chemical estimation of liver glycogen was satis-

factory as determined by control experiments in which known amounts of glycogen were added to pieces of liver.

Stomach contents of fasted mice. Some fasted mice consumed large quantities of the sawdust bedding contained in their cages. When sawdust was excluded, the mean liver glycogen was lower, the "t" test giving $P < 0.001$.

TABLE 2. LIVER GLYCOGEN LEVELS IN FASTED ADRENALECTOMIZED CONTROL MICE

Group	Number of animals	Liver glycogen per 100 gm. mouse mean ±S.E.	Coefficient of variation
1	10	4.679	
2	10	29.166	
3	10	14.309	
4	10	26.470	
5	10	7.210	
6	10	6.830	
7	10	8.628	
8	10	25.590	
9	10	9.328	
10	10	2.026	
11	10	9.498	
12	10	19.474	
13	10	5.475	
14	10	14.925	
15	10	2.781	
1–15	150	12.4 ±1.8	178.0

TABLE 3. COMPARISON OF LIVER GLYCOGEN LEVELS IN FASTED ADRENALACTOMIZED CONTROL MICE KEPT ON DIFFERENT DIETS DURING THE PERIOD OF PREPARATION

Group	Number of animals	Diet	Liver glycogen per 100 gm. mouse mean ±S.E.	Coefficient of variation	*t*	*P*
a	150	stock diet	12.4 ±1.8	178.0	—	—
b	61	diet A+B	12.66 ±2.93	181.25	0.077	>0.9
a+b	211	as above	12.48 ±1.53	178.5	—	—

The coefficient of variation remained very high, however, so that sawdust eating contributed only a little to the scatter of the results.

Fasted mice ate cotton wool, faeces, their own fur and the bodies of dead mice in their cage. In all subsequent experiments mice were kept in special meshwire cages without bedding and with no access to each other. The high coefficient of variation was hardly affected, however, No correlation was established between liver glycogen and gastro-intestinal contents at the time of killing.

Diet and liver glycogen. The 34 control adrenalectomized mice of Venning *et al.* (1946) fasted for 22½ hrs., gave a mean liver glycogen of 4.1 mg./100 g. No mention was made of gross deviations, nor are they suggested by the

small standard error (0.5). The mean liver glycogen of the 242 control mice of Dorfman *et al.*, fasted only 6½ hrs., was 10.8. Large variations were mentioned, some of the control groups averaging as much as 31.5 mg./100 gm. The 28 control mice of Eggleston *et al.* (1946) also fasted for 6½ hrs., averaged less than 5 mg./100 g. and wide variations were not described. In the present investigation, the mean of 150 mice, fasted for 22½ hrs., was 12.4 ± 1.8 with a variation coefficient of 178.0 (Table 2).

Forbes *et al.* (1950) published results of assays with a modified form of Eggleston's method. When mice were fed on the McCollum diet (Diet A), "positive controls," i.e. mice showing more than 9 mg. liver glycogen per 100 gm., occurred frequently. The substitution of bran for whole wheat flour in the McCollum diet (Diet B) resulted in more "positive controls," with values as high as 179.4 mg./100 gm. When "Diet B" was substituted for "Diet A" in the last 15 hrs. before the fast, however, "more rapid emptying of the intestinal tract occurred," and only one "positive control" encountered in a group of 34.

Combined "Diet A+B" was therefore tried in 61 mice, and the results compared with the 150 control mice on stock diet (Table 3). There is no significant difference in the mean or variation coefficient, and the "t" test gives $P > 0.9$.

Liver glycogen levels in control mice do not follow the normal distribution, a fact which may vitiate the application of the "t" test to the two groups. The *means* of samples from abnormal distributions, however, rapidly conform to the normal curve with increase in the size of sample (Emmens, 1948; Finney, 1950). A comparison of the two control groups, based on the mean values of subgroups of 10 mice, is given in Table 4. The "t" test again gives $P > 0.9$.

Temperature and liver glycogen. During the hot summer months, assays gave high results, and liver glycogen in control mice showed striking rises which necessitated the rejection of previous experiments. The effect of temperature was important not only on the day of test but throughout the postoperative period. Temperatures of 76–80°F with little effect in dry weather produce a marked increase in liver glycogen if the atmosphere is humid (Table 5).

Experiments carried out with the object of reducing the error of the assay. Attempts were made to reduce liver glycogen by a variety of procedures in the hope of eliminating "positive controls," even though at the expense of some loss in sensitivity. Forced exercise for 4 hrs.—by the rotating drum method—prior to the assay, and exposure to 4°C for the same period, did not produce the desired result. The possibility of a glycogenic steroid being evolved by the gonads was investigated by performing combined orchidectomy and adrenalectomy on the test mice, but this also gave no better results. A third method was yet tried. Three groups of mice were given

daily subcutaneous injections of 10 g. of l-thyroxine sodium for 4–5 days up to the day of assay. This succeeded in eliminating control values over 10 mg./100 gm. (mean 3.33 ± 0.81), but the coefficient of variation was still unduly high (132.5%), and mice were rendered totally insensitive to Eucortone.

Assay of cortisone. Failure to secure low liver glycogen in control mice and uniform response in mice injected with gluco-corticoids was persistent. It was therefore decided to proceed with the investigation originally planned, employing for the assay the only mice that were available. An assay was carried out with cortisone acetate as a standard (Table 7). A large number of animals, *viz.*, 40 for each dose, had to be used to obtain a reasonably small standard error, and 20 μg represented the smallest

TABLE 4. COMPARISON OF LIVER GLYCOGEN LEVELS IN FASTED ADRENALECTOMIZED CONTROL MICE KEPT ON DIFFERENT DIETS DURING THE PERIOD OF PREPARATION

(Statistics based on mean values of groups, each consisting of about ten mice)

Group	Number of animals	Diet	Liver glycogen per 100 gm. mouse mean ± S.E.	Coefficient of variation	*t*	*P*
a	150	stock diet	12.43 ± 2.3	71.75	—	—
b	61	diet A + B	12.61 ± 2.37	46.0	0.0454	<0.9
a + b	211	as above	12.48 ± 1.80	64.75	—	—

TABLE 5. COMPARISON OF LIVER GLYCOGEN LEVELS IN FASTED ADRENALECTOMIZED CONTROL MICE UNDER DIFFERENT WEATHER CONDITIONS

Group	Number of animals	Temperature range ° F.	Liver glycogen per 100 gm. mouse mean ± S.E.	Coefficient of variation	*t*	*P*
a	211	74 ± 4	12.48 ± 1.53	178.5	—	—
b	91	80 ± 4	70.13 ± 8.03	109.75	9.975	<0.001

TABLE 6. LIVER GLYCOGEN LEVELS IN FASTED ADRENALECTOMIZED CONTROL MICE SUBJECTED TO VARIOUS TREATMENTS DURING THE PERIOD OF PREPARATION

Group	Number of animals	Treatment	Liver glycogen per 100 gm. mouse mean ± S.E.	Coefficient of variation	*t*	*P*
a	211	nil	12.48 ± 1.53	178.6	—	—
b	9	cold—4° C. for 4 hours	9.42 ± 5.43	173.0	0.403	>0.6
c	11	forced exercise	20.50 ± 9.14	147.75	1.116	>0.2
d	9	orchidectomy	37.72 ± 12.27	97.5	—	—
e	30	thyroxine	3.33 ± 0.81	132.5	2.167	<0.05

TABLE 7. LIVER GLYCOGEN LEVELS IN FASTED ADRENALECTOMIZED MICE INJECTED WITH CORTISONE ACETATE ON DAY OF TEST

Group	Number of animals	Dose in μg.	Liver glycogen per 100 gm. mouse mean ± S.E.	Coefficient of variation	*t*	*P*
a	211	0	12.48 ± 1.53	178.5	—	—
b	20	10	17.33 ± 9.13	235.0	0.850	>0.3
c	40	20	28.08 ± 5.33	120.0	1.086	>0.2
d	40	40	52.08 ± 9.54	116.0	2.184	<0.05
e	40	80	105.70 ± 14.12	84.5	3.148	<0.01

amount of cortisone capable of producing a significant difference in response as seen from the "t" test between doses.

The coefficient of variation is again persistently high, though it diminishes in an inverse manner with increase in the mean. A group of fasted

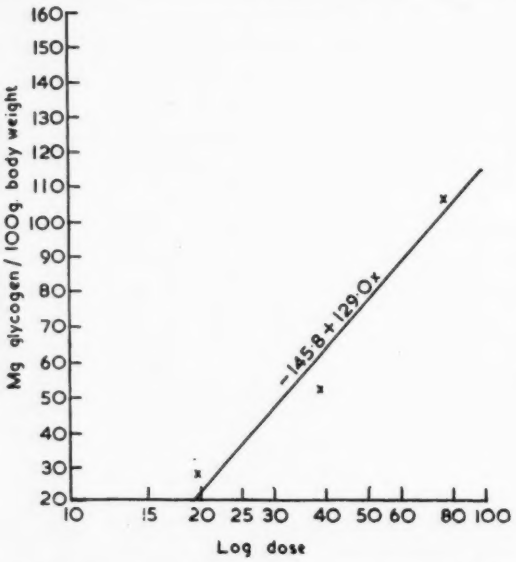

FIG. 1. Logarithm dose-response regression line for cortisone acetate—from 3 points, 20, 40 and 80 micrograms.

normal mice injected with 0.1 ml. *Eucortone* and showing a mean of 46.83 had the same coefficient of variation (116) as the group of adrenalectomized mice injected with 40 μg cortisone with a mean of 52.08. Thus, animals showing comparable means have comparable coefficients of variation in both normal and adrenalectomized groups. This relationship emphasizes again the independence of the high scatter from the technique of adrenalectomy.

The responses to the last three doses of cortisone show a reasonably

good fit to a log-dose repsonse curve (Fig. 1). If the smallest dose (10 μg) is included, the fit is impaired considerably. The reason for this is understood from Figure 2, where all four points are plotted as a linear dose-response curve and fit the regression line almost perfectly. Regression lines are often linear in the lower range of doses of an active substance, and this appears to be the case with cortisone acetate over the range 10–80 μg. The log-dose response curve is used in the comparative studies which follow.

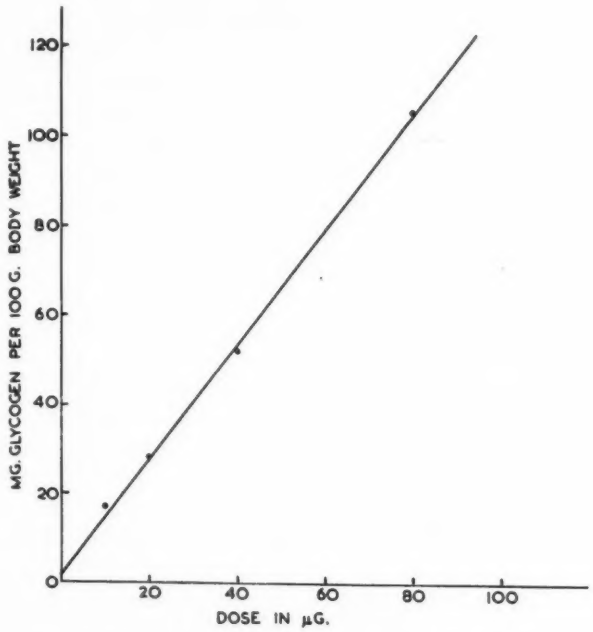

FIG. 2. Linear dose-response regression line for cortisone acetate—from 4 points, 10, 20, 40 and 80 micrograms.

DISCUSSION

The findings of the present investigation are different in several respects from those of other workers, and their careful consideration may result in the saving of time and trouble for many an investigator. These findings have also prompted the critical analysis of the data and conclusions of other authors.

Temperature and liver glycogen. Venning *et al.* (1946) found that during the summer months the "standard dose of 70 mg glucose will cause some deposition of glycogen and consequently the deposition values for known amounts of adrenal corticoid are greater." They recommended that the

sensitivity of mice be checked from time to time with a crystalline gluco-corticoid hormone. The present results indicate that the matter is more serious. Liver glycogen levels in fasted non-adrenalectomized mice which *have not received glucose* show that in hot weather the hepatic glycogen depots are more richly stocked and more resistant to depletion. Many mice show glycogen levels over 200 mg/100 gm without the help of glucose. Unless the animal room is equipped with an automatic cooling device, bioassay experiments must be interrupted during hot weather. A check on sensitivity with a crystalline gluco-corticoid may not be sufficient, since variation among the two groups may be great enough to lead to fallacious conclusions.

The temperatures at which various authors kept their mice were different. Venning *et al.* kept their mice at 76°, Eggleston *et al.* at 78° and Forbes *et al.* at 80±3° F. In the present work, temperatures in the range used by Forbes *et al.* gave high control values (Table 5). The range of 74±4° F. was found to give reasonably reliable results. Whether these differences are ascribed to differences in strain of mice or to other climatic factors, e.g. humidity, it is evident that each investigator will have to find his own optimum range of temperature.

Comparative study of the coefficient of variation. At first the widely scattered values of liver glycogen in control and injected animals were bewildering. Control experiments removed suspicion from the operative procedure and chemical estimation of liver glycogen. With the progress of the work, it became clear that the high coefficient of variation was a characteristic of the mice used. Except for some inverse proportionality with the mean, the magnitude of this coefficient changed little throughout all experiments and was independent of diet, temperature variation, body weight and amount of body fat. To all appearances, therefore, the high coefficient of variation is a constant characteristic of the colony of mice, and is most probably an index of the admixture of strains in that colony.

The above argument is strengthened by the results shown in Table 8, where a comparative study is made of the coefficients of variation of the present experiments and those of other workers. The coefficient of variation is highest in control groups and mostly shows gradual dimunition with increase in the dose of cortisone. The relative magnitude of this coefficient is characteristic throughout the experiments of each investigator, and the average coefficient for the cortisone experiments is given at the foot of the table.

Venning *et al.* made particular mention of the purity of their strain. The coefficient of variation in some of their experiments was as low as 8%. The average coefficient for the whole 354 mice reported in their paper is 35%, and if the control groups are excluded, this falls to 25%. The aver-

age coefficient of variation for the mice of Dorfman *et al.* (1946) is far greater and suggests a fair admixture of strains.

The coefficients of variation were also calculated from the data of Olson *et al.* (1944) and of Pabst *et al.* (1947), who used the glycogen assay method in the rat, and the average figures in their assay of cortisone are 29.57 and 23.25% respectively. Rats are more easily obtained in reliably pure

TABLE 8. COMPARATIVE STUDY OF THE COEFFICIENT OF VARIATION OF THE MOUSE GLYCOGEN METHOD OF ASSAY OF GLUCO-CORTICOID ACTIVITY

(Values calculated from published results of various authors)

Dose of cortisone in μg.	Name of author			
	Nissim	Dorfman *et al.* (1946)	Venning *et al.* (1946)	Eggleston *et al.* (1946)
0 (control)	178.5	101.1	71.0	54.0
10	—	—	23.85	—
20	120.0	75.0	14.5	—
40	116.0	53.75	23.3	—
50	—	—	21.63	—
80	84.5	41.7	—	—
average variation of cortisone	106.825	56.8	20.825	—

TABLE 9. COMPARISON OF VALUES OF COEFFICIENT OF VARIATION, SLOPE, AND INDEX OF PRECISION FOR CORTISONE IN BIOASSAYS CARRIED OUT BY DIFFERENT AUTHORS. THE TABLE ALSO SHOWS PROPORTIONALITY BETWEEN SQUARE ROOT OF MEAN SQUARE OF ERROR(S) AND COEFFICIENT OF VARIATION

	Name of author			
	Nissim	Dorfman *et al.* (1946)	Venning *et al.* (1946)	Eggleston *et al.* (1946)
Average of coefficient of variation for cortisone	106.825	56.8	20.825	—
Slope of line of regression of cortisone (b)	12.9	22.23	9.59	8.1
Index of precision (λ) or s/b	0.505	0.158	0.137	—
Square root of mean square of error(s), calculated from line 2 and 3 above	6.523	3.512	1.314	—
s/v ratio	0.06105	0.0618	0.0631	—

s/v ratio approximately constant.

strains than mice, and it is evident that the accuracy of bioassays carried out with pure strains of rats or mice are of the same order.

The index of precision and the accuracy of bioassay methods. Table 9 presents a comparison of the coefficient of variation, the slope and the index of precision for cortisone bioassays by Dorfman *et al.* (1946) and Venning *et al.* (1946) as well as in the present investigation. The slope represents

the combined sensitivity of method and strain. The index of precision is the square root of the mean square of error divided by the slope, i.e. s/b, named λ by Gaddum (1933). The square of the index of precision is inversely proportional to the amount of information per test object, so that a smaller λ means greater precision.

Dorfman (1950) compared the index of precision of assays by Eggleston *et al.*, Venning *et al.*, and Dorfman *et al.* Eggleston's method D gave the lowest index of precision, and while the indices of Venning *et al.* and Dorfman *et al.* with cortisone were very nearly the same, that of Venning *et al.* was slightly smaller. The precision of the present experiments (0.505) is inferior to all three.

When the square root of the mean square of error (s) is calculated for the three experiments from the slope (b) and the index of precision (s/b), it is seen that they are in strict proportionality with the coefficient of variation (v). The ratio (s/v) gives the values 0.061, 0.062, and 0.063 for the three sets of experiments—an approximately constant figure. In the foregoing analysis it had been concluded that the average coefficient of variation for the cortisone experiments of each of the three workers virtually depended on the purity of the strain of animals used. The proportionality now established between the square root of the mean square of error and the average coefficient of variation shows that the error factor used in the calculation of the index of precision depends in practice on the purity of the strain.

Erroneous conclusions arrived at by using the index of precision (λ) *to compare merit of bioassay methods on animals of same species.* Dorfman (1949, 1950) presented a comparative study of the carbohydrate methods of assay of adrenal cortical hormones based on the value of the index of precision and on the error range of potency ratio. The error range of potency ratio is equal to $s/b \sqrt{1/n_1+1/n_2}$ as a first approximation, and is proportional to the index of precision. Dorfman (1949) concluded that "the method of Venning *et al.* (1946) appears to have the higher accuracy as seen by the fact that a lower error range of potency ratio was found with fewer animals," i.e. because of a smaller λ. Table 9 shows, however, that the lower index of precision obtained for the experiments of Venning *et al.* is due entirely to the smaller mean square of error. The value of the slope, which is proportional to the combined sensitivity of method and strain, is only 9.59 in the method of Venning *et al.* compared with 22.23 in the method of Dorfman *et al.* The calculated greater accuracy of Venning's method is due entirely to the uniformity of the strain. Unless the lower slope obtained by the Montreal workers is attributed entirely to a lower sensitivity of the particular strain of mice, Dorfman's method, as judged from the slope of 22.23, is the more accurate. The confusion of the preci-

sion of experiments with the accuracy of methods has obviously led to erroneous conclusions.

The fallacy resulting from such an assumption is perhaps best illustrated by exchanging the variation of the strain of mice in the two methods. By combining Venning's mean square of error[1] (s^2) with Dorfman's slope (b) and vice versa, the indices of precision would have assumed the following values:

a. Venning's mean square of error and Dorfman's slope, $1.314/22.23 = 0.0591$
b. Dorfman's mean square of error and Venning's slope, $3.512/9.59 = 0.366$

The indices of precision, far from being comparable, would have shown the ratio, $0.366/0.0591 = 6.2$.

By combining Eggleston's mean square of error with Dorfman's slope, and vice versa, even greater differences in λ would have resulted.

Mouse versus rat glycogen methods of assay. Analysis of results led Dorfman to regard the rat glycogen method as "the most precise method available for the bioassay of adrenal cortical material but not the most sensitive." Pabst *et al.* (1947) also found that "the rat gives more reliable results," and pointed out that whereas Dorfman *et al.* (1946) found the error, with groups of 10 mice ($P = 0.05$), to be -59 to $+150\%$, the error in their assays with groups of 10 rats is within 20%. These analyses did not take into account, however, the varied results obtained by different authors with different colonies of mice. In fact, the average coefficient of variation obtained by Venning *et al.* with a pure strain of mice is about the same as that of Pabst or Olson with rats. When this observation is coupled with the established greater sensitivity of mice to cortical hormones it is evident that greater precision should be attained with a pure strain of mice than of rats. Indeed, the index of precision for the assay of cortisone by Venning *et al.* is 0.137 compared with 0.149 for the assay of the same substance by Pabst *et al.* The index of precision for the assay of 11-dehydrocorticosterone by Venning *et al.* (0.222) is, however, higher than that obtained by Pabst *et al.* (0.095) for the same substance. This was mainly responsible for giving a greater error range of potency ratio in the comparison of 11-dehydrocorticosterone to cortisone by the method of Venning *et al.* (-17, $+20$) than that obtained by the method of Pabst *et al.* (-10, $+12$). The numbers of animals used by Venning *et al.* were, however, only 73 compared with 90 used by Pabst *et al.* The average of the coefficients of variation for all the results of Venning *et al.* (263 mice), excepting the control mice, is 25, while the average of this coefficient for the results of Pabst *et al.* with cortisone, adrenal extract and 11-dehydrocorticosterone

[1] The mean square of error (s^2) is the error due to random sampling—in tables of analyses of variance—divided by the corresponding degrees of freedom.

(169 rats) is 34. Even for 11-dehydrocorticosterone itself the coefficient of variation for Venning's mice is 29.07, compared with a coefficient of variation for Pabst's rats of 30.82. Venning's strain of mice, therefore, can give results which are not only more accurate but also at a far smaller level of dosage. The first essential for securing a high level of precision with mice is to ascertain the purity of the strain.

SUMMARY

The mouse glycogen method of Venning *et al.* (1946) was used for the assay of gluco-corticoids, and factors influencing liver glycogen under the conditions of assay were studied. Marked variation in liver glycogen levels of adrenalectomized control and injected mice was noted, and this was paralleled by wide variation in normal mice.

The average coefficient of variation for the cortisone assay was 106.8% as compared with 56.8% for Dorfman *et al.* (1946) and 20.8% for Venning *et al.* (1946). The relative magnitude of this coefficient is characteristic throughout the experiments of each investigator. In the present experiments this was shown to be independent of the adrenalectomy or the chemical assay of glycogen, and is therefore an index of the purity of the strain of animals used.

Erroneous conclusions were arrived at by authors using the index of precision to compare the accuracies of assay methods without attention to differences in the variation coefficient of the different colonies of mice. Variation in strain should not be allowed to confound the issue when the accuracies of methods are compared.

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METHOD OF ASSAY OF A SODIUM-RETAINING FACTOR IN HUMAN URINE[1]

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THE retention of salt and water in a number of clinical conditions has sometimes been attributed to the presence of an excess of a desoxycorticosterone-like substance possibly of adrenal cortical origin. It has also been suggested that other conditions, among them certain rheumatic diseases, may be associated with a relative excess of "mineralocorticoid" (Selye 1951). For some time the validity of these suggestions could not be investigated because a sensitive method for the assay of this material was not available. Dorfman, Potts and Feil (1947) found that the urinary excretion of Na^{24}, during the first 6 hours after its subcutaneous injection in adrenalectomized rats, could be reduced by pretreatment with as little as 1 μg. microgram of desoxycorticosterone-acetate (DCA). A dose-response curve was not given and the effects were not always reproducible. However, the principles suggested by these investigators have been used as a basis for a biological assay procedure by several groups of investigators. None of the modifications, with the exception of that of the present study, have retained the use of Na^{24} as an index of mineral activity. Deming and Luetscher (1950), Spencer (1950) and Marcus, Romanoff and Pincus (1952) measured total urinary sodium by use of a flame photometer, Kagawa, Shipley and Meyer (1952) measured urinary sodium by a microchemical technique, and Simpson and Tait (1952) measured the urinary Na^{24}/K^{42} ratio.

Sodium-retaining activity, as determined by one of the above mentioned methods, has been detected in crude lipid extracts of urine from patients with congestive heart failure and nephrosis by Deming and Luetscher (1950) and Luetscher and Deming (1951), and in the urine of patients with toxemia of pregnancy by Chart, Shipley and Gordon (1951). Studies performed at the University Clinic of the Royal Victoria Hospital, with the method described in this report, have yielded similar results in congestive

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heart failure (Singer and Wener—in press), nephrosis (McCall and Singer —in press) and toxemia of pregnancy (to be published). Although the chemical nature of the active material in these urinary extracts is not known, it is possible that it is similar to a "mineralocorticoid" recently isolated from beef adrenal extract by Grundy, Simpson and Tait (1952) and from adrenal venous blood from a monkey and a dog by Simpson, Tait and Bush (1952). Recently two subtsances have been detected in pregnancy urine with Rf values corresponding with two substances in the amorphous fraction of adrenal cortical extracts (Bush, de Courcey and Gray, 1952).

The present communication includes the description of a method for the assay of a sodium-retaining factor in human urine as well as some studies on the properties of the active material.

METHODS

Assay Procedure

Hooded male rats weighing 160 to 170 gm. at adrenalectomy were used in all experiments. The animals were maintained on a standard diet of Purina fox chow and were adapted to laboratory conditions for one day prior to operation. Room temperature was maintained as close to 80° as possible. The animals were bilaterally adrenalectomized by the dorsal route under light ether anaesthesia and were placed on saline drinking fluid for the first post-operative day and on distilled water for the second. Assays were performed 2 days after adrenalectomy. Rats which had lost 10 grams or more following adrenalectomy, or gained more than a few grams, were discarded. Each group of test animals consisted of 6 to 9 rats. A similar group of control animals was required for each day of testing. The test and control animals used on one day were equally distributed throughout all the cages in order to avoid variations due to the time of handling.

Food and water were removed one hour prior to the injection of the animals. The animals were attached to a board and given subcutaneous injections of the test substance or the solvent vehicle alone (0.1 ml. of absolute alcohol) and 1.0 ml. of an aqueous solution containing 3.5 mg. of sodium chloride with tracer quantities of Na^{24}. The animals were returned to their cages for the urine collection period after ligation of the urethra with heavy thread. Five hours after the administration of the radioactive sodium, the animals were killed by the intraperitoneal injection of Nembutal. The body wall was opened and the bladder exposed. A fine needle and syringe were used to remove the urine directly from the bladder. Bladder and syringe rinses were added to the urine. Aliquots (1.5 ml.) of the urine plus washes were plated onto cupped metal planchets, dried under an infrared lamp and counted in a Geiger counter (Berkeley Scaler Model 1000-B with Tracerlab G-M tube with mica end-window less than 2 mg./cm.^{2}). The mean sodium-24 excretion of the control animals was considered as 100% excretion and that of the test animals was expressed as a percentage of the mean control value. Although the urine assays were all performed in the above manner, studies on individual steroids included the injection of test substances in 0.25 ml. of corn oil or 0.25 ml. of 25% alcohol as well. When the steroids were injected in these two materials, they were given 1 hour before the administration of the radioactive sodium and the urine was collected for a period of 6 hours.

Preparation of Urinary Extracts

Urine was collected without preservative, refrigerated and extracted, generally, within two days. It was acidified to pH 1.5 and extracted three times with redistilled chloroform. The chloroform was then distilled from the residue *in vacuo* at low temperature (water bath temperature 40° C.) and the remaining dry residue dissolved in a small quantity of absolute alcohol and sealed in a glass ampule under light vacuum. The ampules were refrigerated until used. Extracts prepared in this manner are referred to as crude lipid extracts. When desoxycorticosterone was added to normal human urine, which was then extracted as described, recoveries of 85% and 100% were obtained. Neutral lipid extracts were studied on several occasions. These were prepared by washing the crude lipid extract, dissolved in chloroform, with small volumes of 0.1 N NaOH. This was repeated five times. After each washing, the sodium hydroxide was extracted once with an equal volume of chloroform which was then added to the rest of the chloroform extract. The alkali-washed chloroform extract was washed several times with distilled water until the washings were neutral to pH paper. Here again, each washing was back-extracted with chloroform. The neutral lipid extract prepared in this manner was stored in the same way as described for the crude lipid extract. Unless otherwise stated, an amount of extract equivalent to a 20-minute sample of urine was injected into each animal.

RESULTS

Effect of DCA

A sodium-retaining effect was obtained with DCA at all dosage levels studied. The dose-response curves obtained with DCA injected in absolute alcohol (0.1 ml.), 25% alcohol (0.25 ml.) and corn oil (0.25 ml.) are presented in Figure 1. A dosage range of 1 to 20 μg./animal has been stuided. Each point represents the mean value obtained from one to three experiments. The mean Na^{24} excretion of the test animals, expressed as a percentage of that of the control animals, has been plotted against the dosage per rat. Regardless of the differences in solvents and timing, similar curves have been obtained in each instance. When the ratio of the urinary Na^{24} of test to control animals was plotted against the log of the dose, a straight line was obtained for each curve. Significant differences were not observed in the regression coefficients (Tippett, 1941) or the means (Snedecor, 1948) of these lines. The regression line obtained for all studies with DCA is presented in Figure 2. This line is derived from a total of 127 test animals. Significant sodium-retaining effects were observed at the 2 μg. dosage level (P is <0.01) or higher. The free steroid, desoxycorticosterone, has been studied at the 2 and 20 μg. dosage levels and the effect was similar to that observed with the acetate esters at the same dosage levels.

Effect of Cortisone

The effect of cortisone in dosages ranging from 1 to 52.5 μg. per rat is presented in Figure 3. Similar conditions and solvents were used as with DCA.

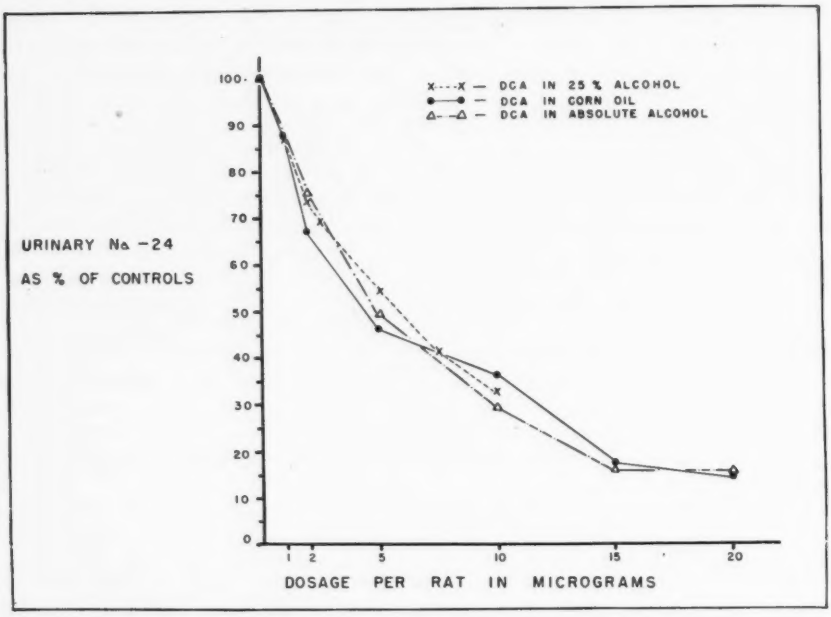

FIG. 1. Effect of DCA on urinary radiosodium excretion in rats. Curve for DCA in absolute alcohol obtained with 5 hour assay. Other curves obtained with 6 hour assay. Na^{24} excretion of control animals was considered as 100% excretion. Values under 100% indicate sodium retention.

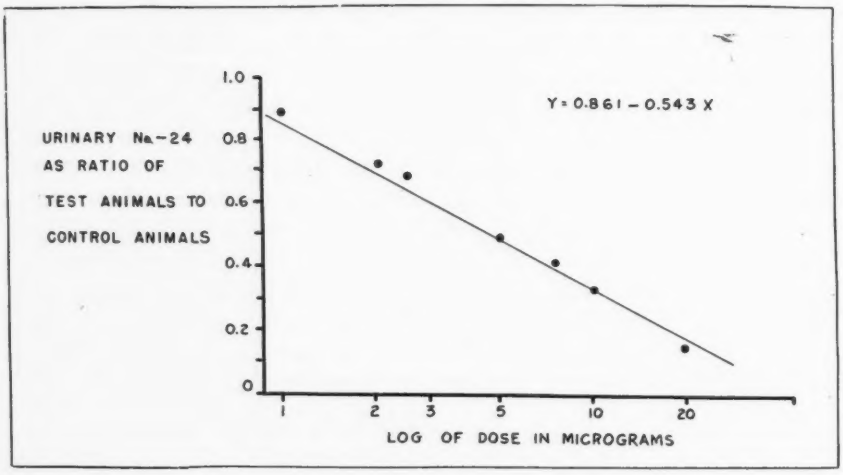

FIG. 2. Effect of DCA on urinary excretion of radiosodium in rats, log-dose-response relation.

Each point in the figure repersents the mean of a single group of 6–9 animals. This has been done because repeat assays at any one dosage of this material did not always give a similar effect. For the most part, animals treated with cortisone excreted more of the injected radioactive sodium than did the control animals, i.e. cortisone had a sodium-excreting effect. Lines have been drawn joining those dosages studied on the same day. Although these results do not give evidence of a clear-cut dose-response relation, they do suggest that the sodium-excreting effect increases with increasing dosage. Sodium-excreting effects were obtained throughout the entire range studied and in both the 6-hour assay (cortisone injected in corn oil and in 25% alcohol) and the 5-hour assay (cortisone injected in

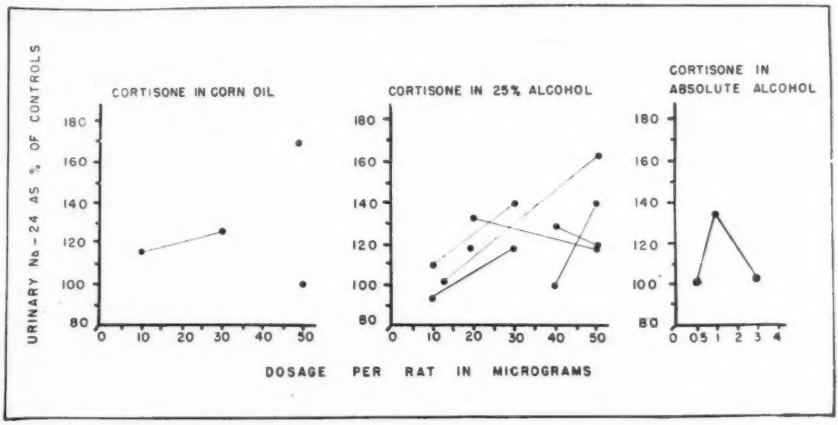

FIG. 3. Effect of cortisone on urinary excretion of radiosodium in rats. Na^{24} excretion of control animals was considered as 100% excretion. Values under 100% indicate sodium retention. Experiments performed on one day are joined by lines. Each point represents the mean value for one group of rats. Studies with cortisone in absolute alcohol were done with the 5 hour assay, the others were done with the 6 hour assay.

absolute alcohol). A sodium-retaining effect was observed on only two occasions and even then was not significant since the test animals excreted over 90% as much Na^{24} as the control animals.

Effect of Storage on the Activity of Urinary Extracts

Since the assays with radioactive material could not always be carried out immediately after the collection and extraction of the human urine, storage of the extracts for periods of several weeks was sometimes necessary. It was observed that a number of the extracts, which had been stored in the dry state at room temperature, lost a great deal of their sodium-retaining effect in 2 to 4 weeks. The change in activity of 4 extracts

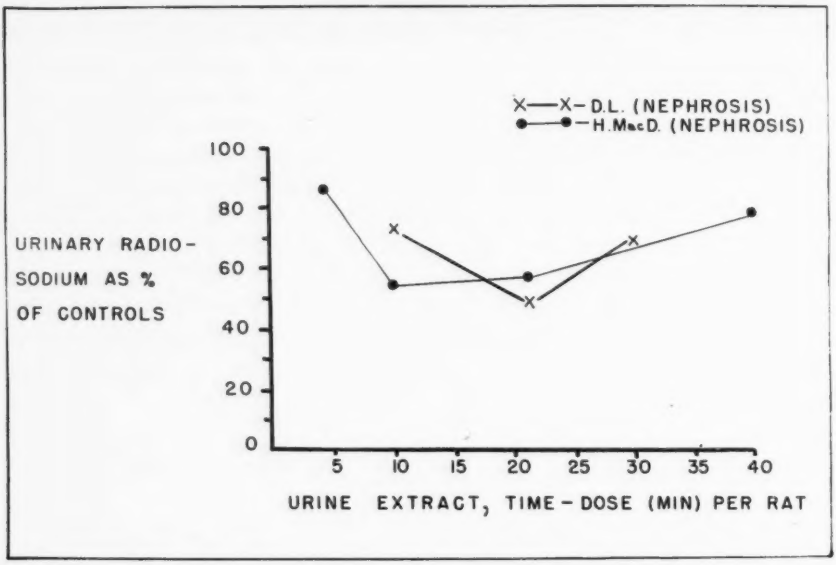

FIG. 4. Changes in activity of urinary extracts with time: effect of different methods of storage. Radiosodium excretion of control animals was considered as 100% excretion. Values under 100% indicate sodium retention.

stored in this manner (referred to as "dry" storage) for several weeks is presented in Figure 4. The mean urinary radiosodium of the test animals, as a percentage of that of the control animals, has been plotted against the age of the extract in days. Losses in activity were observed in 3 of the 4 extracts. When the extracts were sealed in ampules in small amounts of absolute alcohol and refrigerated until assayed, losses during storage were negligible. Studies with three such extracts, stored for various periods of time, are presented in the same Figure. This method of storage is referred to as "wet" storage and was adopted for routine purposes.

Effect of Alkali and Water Washing on Activity of Urinary Extracts

Two urinary extracts obtained from patients with nephrosis were studied in the crude lipid form and in the neutral lipid form, i.e. after washing of the crude lipid fraction with dilute alkali and water. Results are presented in Table 1. These two studies suggest that the active material is relatively alkali-insoluble since recoveries of 114.3% and 81.7% were obtained in the washed chloroform fraction.

Effect of Variations in Time-dosage on Activity of Urinary Extracts

Two crude lipid extracts obtained from the urine or patients with nephrosis were studied at time-dosages ranging from 4.4 to 40 minutes per

TABLE 1. EFFECT OF ALKALI AND WATER WASHINGS ON SODIUM-RETAINING ACTIVITY OF CRUDE LIPID URINARY EXTRACTS[1]

Subject	Fraction	No. test rats	No. control rats	Urinary Na^{24} (% of controls)[2]	Approx. DCA equivs. (μg.)	
H.MacD.	Crude	5	7	57.1		4.4
H.MacD.	Neutral	6	8	50.0		4.9
					Recovery	114.3%
D.L.	Crude	8	10	49.7		4.9
D.L.	Neutral	6	6	58.5		4.0
					Recovery	81.7%

[1] Studies performed on urine of patients with nephrosis.
[2] Na^{24} excretion of control animals was considered as 100%. Values under 100% indicate sodium retention. DCA equivalents taken from curve obtained with DCA in absolute alcohol.

rat (Fig. 5). Results indicated that the sodium retaining effect was inhibited at the higher dosage levels. In one case (H.MacD.) studied at the 4.4, 10, 20 and 40 minute dosage, a maximum effect was reached at the 10 minute level. This degree of activity was maintained at the 20 minute dos-

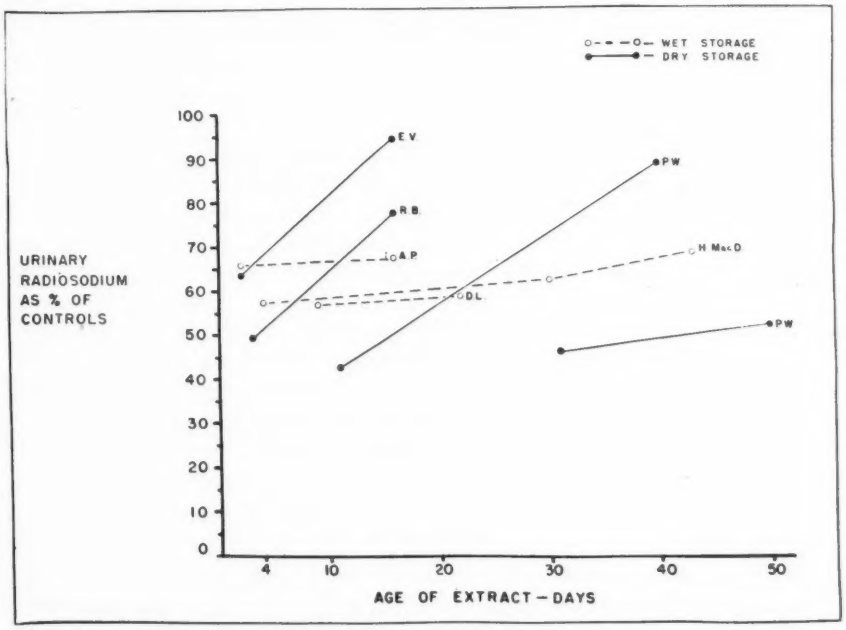

FIG. 5. Effect of different time-dosages of crude lipid extracts of urine on radiosodium excretion in rats. Na^{24} excretion of control animals was considered as 100% excretion. Values under 100% indicate sodium retention.

age but decreased again at the 40 minute level. The second extract (D.L.) which was studied at the 10, 20 and 30 minute dosage levels, showed a maximum sodium-retaining effect at the 20 minute level. The effects at the 10 and 30 minute dosages were similar.

Comparison of Method of Deming and Luetscher with Present Method

Dr. Deming and Dr. Luetscher kindly forwarded samples of two crude lipid extracts obtained from the urine of patients with nephrosis for comparison with the present assay. They were prepared by chloroform extraction of urine acidified to pH 1.5 immediately prior to extraction and stored in alcohol until assayed. Results obtained on these two extracts by Drs. Deming and Luetscher and by the present assay procedure are presented in Table 2. The extracts had marked sodium retaining effects in both assay procedures. Although some variation in the methods have been noted, results indicate that the order of sensitivity is within the same range in both methods. One extract, No. 4401, was more active in the assay described in this communication than it was in the laboratory of Deming and Luetscher (i.e. 10 μg. of DCA equivalents as compared with 6.2 μg.). Extract No. 2216 had less activity in the present assay than was observed by Deming and Luetscher (i.e., 4.3 μg. of DCA equivalents as compared with 7.3 μg.) but, since this extract was rather old when studied by the present method, this was not unexpected.

TABLE 2. SODIUM-RETAINING ACTIVITY OF TWO URINARY EXTRACTS BY THE METHOD OF DEMING AND LUETSCHER AND THE PRESENT ASSAY

Subject	Age of extract	No. test rats	No. control rats	Urinary Na^{24} (% of controls)[1]	Approx. DCA equivs. (μg.)
Results obtained by Deming and Luetscher[2]					
2216					7.3
4401					6.2
Results obtained in present assay					
2216	13.5 mos.	13	10	56.1	4.4
4401	1.0 mos.	14	14	27.6	10.0

[1] Na^{24} excretion of control animals was considered as 100%. Values under 100% indicate sodium-retention. DCA equivalents were taken from the curve obtained with DCA in absolute alcohol.

[2] Personal communication Dr. Q. B. Deming, November 6th, 1951.

DISCUSSION

A method for the detection of sodium-retaining material, based on principles suggested by Dorfman *et al.* (1947), has been standardized as a method for the bio-assay of sodium-retaining substances in human urine. The assay procedure is similar to those recently developed by several groups of investigators, and has a similar range of sensitivity to those

methods employing adult rats (Deming and Luetscher, 1950; Kagawa, Shipley and Meyer 1952; and Marcus, Romanoff and Pincus 1952) although it is slightly less sensitive than those methods employing mice (Spencer 1950) or young rats 30 to 40 gm. (Simpson and Tait 1952). The similarity of the present assay with that of Deming and Luetscher is further illustrated by the fact that two urinary extracts had similar sodium-retaining effects in their hands and in ours.

In order to obtain reproducible results with DCA in the present assay, constant environmental conditions, weight of animals, pre and postoperative treatment as well as a constant procedure during the assay period were found to be essential. An important feature of this assay is that results must always be expressed in relation to control animals studied on the same day, as these do vary somewhat from day to day regardless of efforts to obtain constant conditions. All of the recently developed methods for the assay of substances affecting mineral metabolism stress the necessity of maintaining rigid conditions and most of them express results in relation to control animals studied on the same day (Simpson and Tait 1952, Kagawa *et al.*, 1952) or in relation to values obtained in the same animals serving as controls (Spencer 1950, and adopted later by Luetscher and Deming 1951). It is apparent from the present study, as well as those of others, that the excretion of sodium in adrenalectomized animals is quite variable and that for best results a group of at least eight animals should be used for each unknown.

The fact that cortisone generally has a sodium excreting effect in the assay, compared with that of DCA, introduces some difficulty in the interpretation of negative results in assays of urinary extracts, since these extracts also contain cortisone-like material. This effect of cortisone may have some bearing on the fact that a decreasing sodium-retaining effect is observed with increasing time-dosages, although the effect of other substances has not been ruled out. Until more highly purified extracts possessing a more consistent dose-response relation can be prepared, absolute results in urinary extracts must be evaluated with caution.

With regard to the possible interfering effect of cortisone in the assay, it would appear that the use of the urinary Na^{24}/K^{42} ratio, as described by Simpson and Tait (1952), may be useful in studying this effect. In their method, all the adrenal steroids tend to reduce the ratio in varying degrees. Thus, if a urinary extract contained both cortisone-like and "mineralocorticoid" material, one might expect that the effects would not cancel each other out but would, in fact, be additive. This point has not, however, been investigated. The quantity of cortisone-like material present in an extract could then be determined by other means if one were interested in knowing whether a separate "mineralocorticoid" were present in the unknown. The

difference in the effect of cortisone in the present assay and that of Simpson and Tait is probably related to the fact that these investigators study the change in the ratio of Na^{24} to K^{42}, whereas the present method is based on the Na^{24} excretion alone. It is quite possible that cortisone may increase the urinary excretion of both of these substances but that the effect on potassium may be slightly greater, resulting in the observed fall in the ratio. The short urine collection period (2 hours) plus the low salt load administered to the animals by these investigators may also have some bearing on the effects of cortisone in their assay.

Since the chemical nature of the sodium-retaining factor in urine is not known, the reason for the apparent destruction of this material following "dry" storage cannot, at the moment, be ascertained. In this connection, it may be of interest that certain common molds can be used for the oxygenation of desoxycorticosterone, 17-hydroxy-11-desoxycorticosterone and progesterone (Colingsworth, Brunner and Haines 1952, and Peterson and Murray 1952). If the sodium-retaining factor in urine is a desoxy compound of this type, it is possible that this kind of reaction may have been responsible for the lability of extracts which were stored in the "dry" state at room temperature. The fact that losses were considerably smaller after storage in absolute alcohol with refrigeration lends some support to the suggestion that the decrease in activity of urinary extracts may have been due to a microbiological agent.

SUMMARY

A method for the detection of small quantities of sodium-retaining material has been standardized and applied to the study of crude lipid extracts of human urine. The method is based on the excretion of a dose of Na^{24} by adrenalectomized animals in a 5 hour period.

DCA had a sodium-retaining effect in the dosage range of 1 to 20 μg. per animal. A linear relation was observed between the log of the dose and the response. Significant sodium retention was obtained at the 2 μg. dosage level and higher.

Cortisone had mild to marked sodium-excreting effects which were not always reproducible, in the range of 1 to 52.5 μg. A consistent dose-response relation was not observed.

Urinary extracts could be stored for periods of at least 6 weeks without serious loss in activity if they were dissolved in alcohol, sealed in ampules and refrigerated until assayed.

The sodium-retaining effect of two crude lipid extracts of urine were not significantly altered by dilute alkali and water washings.

Dosage-response curves of two crude lipid urinary extracts indicated the

presence of interfering substances at the 20 minute level in one case and the 30 minute level in the other.

Acknowledgments

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EFFECT OF STIMULATION OF THE REPRODUCTIVE ORGANS OF THE COW ON THE RELEASE OF AN OXYTOCIN-LIKE SUBSTANCE

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FOR many years it has been recognized that there is a close association between the reproductive system and the mammary gland, however the physiological connection between the two has not been well defined. Several reports in the literature suggest that the ejection or "let-down" of milk in the mammary gland can be caused by the stimulation of the reproductive organs. This is not entirely a neural connection since there can be an ejection of milk even when the efferent nerves to the mammary gland have been cut (Ingelbrecht, 1935; Ely and Petersen, 1941; Espe, 1947). Generally it is accepted that the principal cause of milk ejection in the intact animal is the action of oxytocin on the mammary gland.

The purpose of this study was to determine the effect of stimulation of the reproductive organs of the cow by artificial insemination techniques and natural mating on the release of oxytocin as measured by increased intramammary pressure.

REVIEW OF LITERATURE

Some evidence suggesting a relationship between stimulation of the reproductive organs and milk ejection is found in the work of Nuesch (1904). In a review of literature, he states that a publication in 1727 refers to a practice of the Hottentots of blowing air into the vagina to bring about milk ejection in the absence of the calf. Nuesch also states that the Skyths of India milked mares in a similar manner. He further states that the ejection of milk in the cow can be brought about by rinsing the vagina or uterus, blowing air into the vagina, or even the manual removal of afterbirth. Tgetgel (1926) reported that manual manipulation of the uterus and ovaries of a cow caused milk ejection as did the presence of another cow in estrus. It has also been noted in our work that palpation of the uterus caused milk to drop from the teats. Hammond (1936) made the observation that in lactating mares, milk often flows freely during mating.

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METHODS AND MATERIALS

The experimental animals were 17 non-pregnant cows which were in their first to eighth lactation and were producing 20–67 pounds of milk daily. The experiments were done 7–11 hours after milking to allow time for milk to accumulate in the udder. The intramammary pressure was measured by a water manometer connected to a teat cannula which was inserted into the teat or gland cistern. Readings were taken with the cows in the same position each time since the position of the legs in reference to the udder is an important factor in intramammary pressure. If there was an ejection of milk due to insertion of the test cannula, a period of 20–30 minutes was allowed to elapse so that the pressure would return to normal or near normal.

In a preliminary study, 8 cows were tested at various times during the estrous cycle. Trials were made on 9 additional cows on the following days of the cycle: 1, 2, 4, 12, 16, 18, and 20 with the day of estrus being considered as day 1. The intramammary pressure was noted at one-minute intervals during massage of the vulva, massage of the cervix and cervical os through the rectal wall and during the insertion of a 7–8 mm. glass inseminating tube into the cervix. Also the pressure was noted at intervals after natural mating on the day of estrus.

In order to prove that oxytocin or an oxytocin-like factor was present in the blood after stimulation, three pairs of blood samples were assayed with different excised cow uteri as test organs. The uterus was kept in a bath of Tyrode's solution at 39° C. and perfused with Tyrode's solution. To the perfusate was added samples of approximately 250 cc. of blood, taken before and after stimulation of the vulva, cervix, and uterus by palpation. The motility was determined by inserting into the excised uterus a balloon which was connected to a tambour. The contractions were recorded by a kymograph.

RESULTS

In 99 trials on 16 cows the artificial insemination techniques caused an increase in intramammary pressure in 88% of the trials. An increase of 1.5 cm. or more of water was considered a positive response. The trials which gave a positive response showed an average increase in pressure of 8.0 $\pm$ a standard error of the mean of 0.62 with a range of 1.5 to 31 cm. of water. Every cow gave a positive response in some or all of the trials. The results of the experiment on the 9 cows are shown in Table 1. The difference due to cows was highly significant. The differences between responses on different days of the cycle were not significant.

The increase in pressure following the different stimuli varied between cows and from time to time in the same cow. The response was caused by stimulation of the vulva, stimulation of the cervix by massage or stimulation of the cervix by the inseminating tube. In many trials the total response was due to a combination of two or more of the above stimuli. In 15 natural services 80% gave a positive response for an average increase in pressure of 4.5 cm.

Blood taken before stimulation of the vulva and cervix by massage had no effect on uterine motility when perfused into the excised uterus. But perfusion of the three blood samples taken after stimulation caused the

TABLE 1. INTRAMAMMARY PRESSURE INCREASES (CM. OF WATER) AFTER STIMULATION OF THE REPRODUCTIVE ORGANS

Cow	Day of the estrous cycle							Ave.
	1	2	3–4	12	16	18	20	
1	8	0	5	3	4	7	2	4.1
2	5	7	5	6	5	2	4	4.9
3	2	3	4	0	5	2	3	2.7
4	13	20	16	14	7	6	7	11.9
5	4	7	8	12	16	2	6	7.9
6	11	0	8	15	2	4	6	6.6
7	0	4	12	4	7	12	8	6.7
8	31	17	18	22	13	10	12	17.6
9	2	6	0	5	2	6	4	3.6
Ave.	8.4	7.1	8.4	9.0	6.8	5.7	5.8	7.3

uteri to have a series of contractions. The motility pattern was very similar to that caused by perfusion of oxytocin.

DISCUSSION

The positive response of increased intramammary pressure indicates that stimulation of the reproductive organs of the cow causes milk ejection. The nerve impulses initiated by the stimulation conceivably cause the release of a hormone which brings about the ejection. Since it has been shown that cutting the nerve supply of the mammary gland does not interfere with normal milk ejection (Ingelbrecht, 1935; Ely and Petersen, 1941; Espe, 1947) and that stimulation of the known nerves to the udder does not cause milk ejection, the possibility of reflex action is remote. The facts that the responses occurred in 10–30 seconds after stimulation and that the effective substance is released into the blood after stimulation indicate that it is a hormone. It is generally accepted that the principal cause of milk ejection is oxytocin (Gaines, 1915; Ely and Petersen, 1941; Petersen and Ludwick, 1942). This combined with the fact that perfusion of blood taken after stimulation into the excised uterus gave a response very similar to that caused by oxytocin indicates that the hormone is oxytocin.

It is noteworthy that there is a reciprocal relationship between the reproductive organs and the mammary glands by the medium of nerve impulses and oxytocin. Proper stimulation of the reproductive organs or the mammary glands causes the release of oxytocin which in turn causes contractions in both the mammary glands and reproductive organs.

The responses due to stimulation of various sites of the reproductive tract showed that the sensory receptors were mainly in the vulva and cervix, and were especially prevalent in the area of the external os of the cervix. The responses during natural mating probably indicate that these receptors were stimulated during the act of mating.

Since the differences in response due to the different parts of the estrous cycle were not significant, the responses did not vary with changes in the levels of the female sex hormones. Two factors seemed to play a role in the highly significant differences in responses of the individual cows. First, the largest net increases in intramammary pressure were observed in those animals which exhibited the highest levels of pressure after stimulation. Another factor was that in most cases, but not all, the more nervous cows, as judged by their outward reactions, did not show as great a reaction as the more quiet ones. This could possibly be explained by the fact that the nervous cows released epinephrine which tended to counteract the effect of oxytocin on the mammary gland. This counteraction by epinephrine has been shown to occur (Ely and Petersen, 1941).

Recent studies on the motility of the bovine uterus indicate that the oxytocin like factor is released at the time of natural mating due to the stimuli of the presence of the bull, mounting by the bull or copulation (VanDemark and Hays, 1952) and during artificial insemination due to the manipulation of the reproductive organs (VanDemark and Hays, 1951).

SUMMARY

Stimulation of the vulva and cervix by massage caused an increase in intramammary pressure in 88% of 99 trials on 16 cows for an average increase of 8.0 cm. of water. In 15 natural matings, 80% gave a positive response for an average increase of 4.5 cm. of water. Blood taken before massage of the vulva and cervix had no effect, but blood taken after stimulation caused contraction of excised, perfused cow uteri in each of 3 trials similar to that caused by oxytocin. It is postulated that stimulation of the reproductive organs of the cow causes the release of oxytocin.

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A COMPARISON OF THE EFFECTS OF CORTISONE ADMINISTERED INTRAPERITONEALLY AND SUBCUTANEOUSLY IN THE RAT

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IT IS well known that the route of administration greatly modifies the effects of many steroids. Estradiol benzoate, testosterone propionate, and desoxycorticosterone acetate, for example, are very effective when administered parenterally but quite ineffective when administered orally, presumably due to the inactivation of these substances by the liver. On the other hand, it has been known that oral administration of whole adrenal extract is metabolically effective since 1937 when Ingle and Kendall demonstrated that atrophy of the adrenal cortex was produced by the feeding of whole adrenal cortical extract (ACE) to rats in the drinking water. Since then it has been amply confirmed that oral administration of whole adrenal extract in drinking water (D'Amour and Funk, 1937; Ingle, 1938; Ingle and Higgins, 1938; Ingle, Higgins and Kendall, 1938), oral or subcutaneous corticosterone (Wells and Kendall, 1940), and oral, subcutaneous or intramuscular cortisone (Ingle and Mason, 1938; Wells and Kendall, 1940; Antopol, 1950; Stebbins, 1950; Winter, Silber and Stoerk, 1950; Lewis, Rosemberg and Wilkins, 1950; Tuchman-Duplessis, 1951; Higgins, Woods and Kendall, 1951; Migeon, *et al.*, 1952; and Halmi and Barker, 1952) all produced inhibition of growth and thymic and adrenal atrophy in rats and mice. It is of interest that Howard and Grollman (1934) were unable to produce growth inhibition, or significant effects on the adrenals by the administration of whole adrenal cortical extract to rats intraperitoneally, but it was not clear whether this was due to the route of administration or to the lack of potency of the extracts. However, with all of the evidence of the marked metabolic effects of ACE and cortisone when administered orally or parenterally, we were quite surprised to find, in the work to be reported, large amounts of cortisone could be given to rats intraperitoneally without significant inhibition of growth, and without producing atrophy of the thymus or adrenal cortex. Yet we were able to demonstrate that the intraperitoneally administered cortisone was metabolically active.

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MATERIALS AND METHODS

Albino rats of the Stanford Colony were maintained on a stock diet (Addis and Gray, 1950), and tap water *ad libitum*. Cortisone Acetate,[1] suspended in standard diluent, was injected intraperitoneally (i.p.) or subcutaneously (s.c.) daily. Control animals received either saline or diluent[2] i.p. or s.c. daily. In the first series of 30 female animals (Table 1, Groups 1–4) the cortisone-treated animals were injected as follows: Cortisone acetate 1.25 mg. daily for 14 days, 2.5 mg. q.d. for 14 days, and 3.75 mg. q.d. for 20 days, a total of 127.5 mg. per rat over a period of 48 days. In the second series of 20 male animals (Table 1, Groups 5–8), the treated animals received cortisone acetate 3.75 mg. daily for 40 days, a total dose of 150 mg. per rat. Twenty-four hours after the last injection, the animals were anesthetized with ether, exsanguinated, the organs weighed on a torsion balance and fixed in Bouin's solution.

RESULTS

It was noted during the experiment that the animals injected with cortisone s.c. failed to gain weight and actually lost weight, whereas the animals injected with cortisone i.p. could not be distinguished from the controls. At autopsy, we found large deposits of unabsorbed cortisone under the skin of the animals injected s.c., but no trace of unabsorbed cortisone in the animals injected i.p.

TABLE 1. THE EFFECT OF SUBCUTANEOUS AND INTRAPERITONEAL CORTISONE ON THE BODY WEIGHT, THYMUS WEIGHT AND ADRENAL WEIGHT OF THE RAT

Group	No. rats, sex	Material and route administration	Initial body weight, gm.*	Final† body weight, gm.*	Thymus weight, mg.*	Adrenal weight, mg.*
1	10 F	Saline i.p.	157 (7.7)	191 (14.1)	221.5 (48.7)	44.6 (5.6)
2	10 F	Cortisone i.p.	165 (7.1)	206 (9.2)	180.8 (45.9)	39.7 (5.0)
3	5 F	Diluent s.c.	159 (4.1)	197 (5.6)	207 (17.4)	50.2 (6.4)
4	5 F	Cortisone s.c.	163 (6.3)	149 (3.9)	0	15.2 (3.0)
5	5 M	Diluent i.p.	234 (7.3)	289 (13.5)	205 (39.9)	31.3 (4.5)
6	5 M	Cortisone i.p.	230 (12.8)	263 (30.7)	155 (38.6)	27.4 (1.0)
7	5 M	Saline s.c.	280 (21.3)	333 (28.9)	235 (35.4)	33.8 (3.3)
8	5 M	Cortisone s.c.	275 (22.1)	240 (30.9)	0	14.0 (6.5)

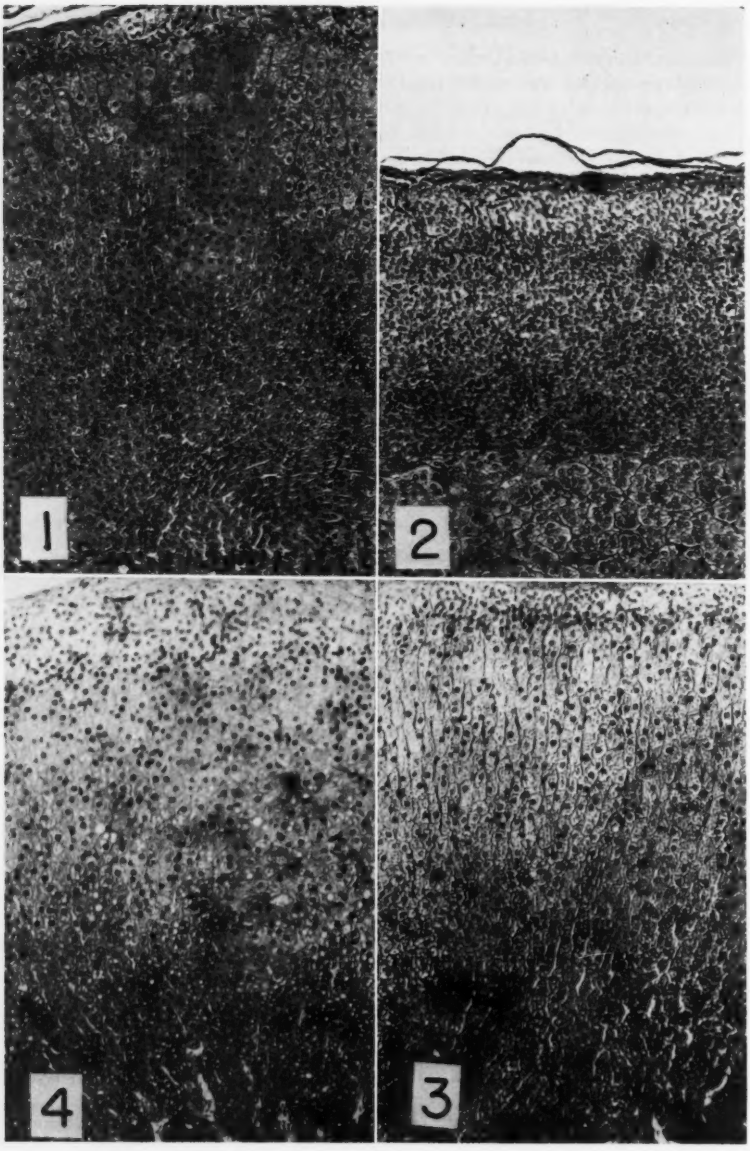
* Figures in parentheses are the standard deviation of the mean, corrected for small groups.

† Groups 1–4 treated for 48 days; Groups 5–8 treated for 40 days.

A comparison of the effects of cortisone injected s.c. and i.p. on the body weight, thymus weight and adrenal weight is presented in Table 1. In the first series of female animals, during the 48 days of the experiment, control Groups 1 and 3 gained an average of 36 gm. per rat, Group 2 (cortisone, i.p.) gained 41 gm., whereas Group 4 (cortisone, s.c.) lost an average of 14 gm. per rat. The thymus weights of the animals in Group 2 (cortisone i.p.) were not significantly different from the controls, whereas in Group 4

[1] Cortone, (R), Merck.

[2] The Cortone (R) Diluent was kindly supplied by Merck and Company, Rahway, New Jersey.



FIGS. 1–4

(cortisone, s.c.) no thymic tissue could be found at autopsy. The adrenal weights of Group 2 averaged 39.7 gm., which was not significantly different from the controls. The adrenals in Group 4 (cortisone, s.c.) weighed an average of only 15.2 mg., which is a highly significant difference from the adrenal weights of the controls. Cortisone had no effect on the weights of thyroid, ovary, uterus or hypophysis in either of the treated groups.

In the second series of male animals, Group 5 served as a weight control for Group 6 (cortisone, i.p.) and Group 7 served as a control for Group 8 (cortisone, s.c.). Again we found that Group 6 (cortisone, i.p.) gained an average of 33 gm. per rat per 40 days, which was not significantly different from the weight gain of the control group, whereas Group 8 (cortisone, s.c.) lost an average of 35 gm. per rat. The thymus and adrenal weights of Group 6 did not differ significantly from the controls, whereas there was no discernible thymic tissue in the animals of Group 8 (cortisone, s.c.) and the adrenals of this group were only 14 mg., compared to 33.8 mg. for the adrenal of the controls. Cortisone by either route of administration had no effect on the weights of the thyroid, testes, seminal vesicles, prostate or hypophysis.

Histologically, cortisone administered s.c. produced marked atrophy of the fascicular and reticular zones of the adrenal cortex while the glomerular zones remained relatively intact. This occurred in both male and female animals. Representative sections from the adrenals of these female animals are reproduced in Figures 1–4. The changes produced by cortisone s.c. are similar to those reported by Greep and Deane (1949), Yoffey and Baxter (1949), and Stebbins (1950). Cortisone i.p., however, produced no significant change in the histology of the adrenal cortex. We found no significant changes in the histology of the thyroids, ovaries or testes of either cortisone treated groups. We were unable to find that injection of the diluent produced any pathological changes in the endocrine organs of either sex.

EXPLANATION OF FIGS. 1–4

FIG. 1. Adrenal of female rat No. 68 (Group 3) receiving 0.15 ml. of Cortone® diluent subcutaneously, daily for 48 days. The three zones of the cortex are of normal width. (×100)

FIG. 2. Adrenal of female rat No. 58 (Group 4) receiving a total of 127.5 mg. of cortisone acetate subcutaneously over a period of 48 days. There is marked decrease in width of the cortex, due primarily to a reduction of the zona fasciculata. It is not possible to clearly define the margins of the zona fasciculata and zona reticularis. A portion of medulla shows at the bottom of the picture. (×100)

FIG. 3. Adrenal of female rat No. 82 (Group 2) receiving a total of 127.5 mg. of cortisone acetate intraperitoneally over a period of 48 days. Cortex of normal width with three distinct zones. (×100)

FIG. 4. Adrenal of male rat No. 20 (Group 6) receiving a total of 150 mg. cortisone acetate intraperitoneally over a period of 40 days. Cortex of normal width with 3 distinct zones (×100).

DISCUSSION

From the above data it was clear that cortisone administered intraperitoneally to the rat produced neither cessation of growth, thymic atrophy nor adrenal atrophy. There seemed to be several possibilities to explain this. First, the cortisone might not have been absorbed intraperitoneally. This did not seem likely since we were unable to find any unabsorbed material in the peritoneal cavity of the animals injected i.p., whereas despite the finding of a relatively large amount of unabsorbed material under the skin of the animals injected s.c., these animals showed marked effects. Second, the intraperitoneal cortisone might be inactivated in some fashion. This was possible, but did not seem likely because of the known effectiveness of cortisone orally and the failure of the liver to inactivate the adrenal hormones produced by the adrenal transplanted to the spleen (Bernstein, 1950) or cortisone pellets placed directly in the spleen (de Andino and Rivero-Fontan, 1951). Further, Mello (1949), showed increased liver glycogen deposition, increased survival and increased resistance to cold following intraperitoneal administration of whole adrenal cortical extract to adrenalectomized rats, and Speirs (1951) has reported eosinopenia in adrenalectomized mice following i.p. cortisone. There is little doubt, therefore, that cortisone is not inactivated intraperitoneally. Why does it not produce metabolic effects comparable to subcutaneously administered cortisone? The third possibility is that i.p. cortisone may be absorbed so rapidly that it does not maintain a blood level long enough or high enough for maximum metabolic effects, such as growth inhibition or adrenal atrophy. Some basis for this thesis is found in the observation of Wilkins, *et al.* (1952) on the increased effectiveness of intramuscular over oral cortisone therapy in supressing adrenal function in congenital adrenal hyperplasia in children. It was therefore of importance to determine the intensity and duration of the effect of a single dose of cortisone administered s.c. and i.p. in rats.

To test the effect of a single large dose of cortisone in the rat, a bioassay was devised utilizing the principle of the restoration of water diuresis in the adrenalectomized rat, as suggested by Eversole, Gaunt and Kendall (1942). The procedure was as follows: 15 female albino rats weighing 150–160 gm. were adrenalectomized, divided into three groups of 5 animals each and maintained on a stock diet with 1% saline as drinking water. The assay was begun 3 days postoperatively. On the control day of the assay, the animals received a hydrating dose equal to 2.5% of their body weight of distilled water by stomach tube. Three hours later they received a second dose of 5% of their body weight of distilled water by stomach tube. They were then placed in metabolism cages and the urine output recorded every 30 minutes for three hours. On the day following the control run, at the time of the initial hydration (three hours before the measurement of their

diuretic response to a water load) one group of 5 animals received 3.75 mg. of cortisone s.c., a second group of 5 animals received 3.75 mg. cortisone i.p., and the third group of 5 animals received 0.15 cc. saline s.c. The animals were then given the same dose of water by body weight for the next four days and the urine output determined. No further cortisone was administered. All animals were maintained on 1% saline at night. By the fifth day of water hydration the adrenalectomized control animals had become quite ill, but both cortisone treated groups looked well.

On each successive day following the administration of cortisone or

TABLE 2. THE EFFECT OF CORTISONE INTRAPERITONEALLY AND SUBCUTANEOUSLY ON THE DIURETIC RATIO* IN ADRENALECTOMIZED RATS SUBJECTED TO A DAILY WATER LOAD†

Hours after injection	Untreated controls	Cortisone s.c.	Cortisone i.p.
Control	1.00	1.00	1.00
3	.594	1.16	3.42
27	.565	1.25	2.00
51	.189	1.10	1.15
75	.087	2.18	1.13
99	0	1.06	.68

* Diuretic Ratio = $\frac{\text{Mean 90 min urine output given period}}{\text{Mean 90 min urine output control period}}$.

† See text for details.

saline, the total urine output per animal per 90 minute period, corrected for the weight of the animal, was divided by the output during the control period to obtain a "Diuretic Ratio" as follows:

$$\frac{\text{Mean 90 minute urine output given period}}{\text{Mean 90 minute urine output control period}} = \text{Diuretic Ratio.}$$

A Diuretic Ratio greater than 1 means a 90 minute diuresis greater than that during the control period, whereas a Diuretic Ratio of less than 1 means a diuresis less than during the control period.

The results of this study are shown in Table 2 and Figure 5. The untreated adrenalectomized animals put out less and less urine following repeated hydrations until at 99 hours the Diuretic Ratio fell to zero. The animals which received cortisone i.p. responded to a water load 3 hours after receiving cortisone with a large diuresis, so that the Diuretic Ratio at 3 hours after the injection rose to 3.42. Thereafter, the Diuretic Ratio fell slowly to about 0.7 at 99 hours. The animals which received cortisone s.c. maintained a constant Diuretic Ratio about 1 (i.e., the 90 minute urine output remained equal to the control day of the untreated adrenalectomized animals) until 75 hours when the Diuretic Ratio rose to 2.2, after which it fell at 99 hours to about 1.

These data would suggest that cortisone i.p. has an immediate effect on the restoration of water diuresis in the adrenalectomized rat. This effect is maximal on the day of injection, and gradually diminishes over the next

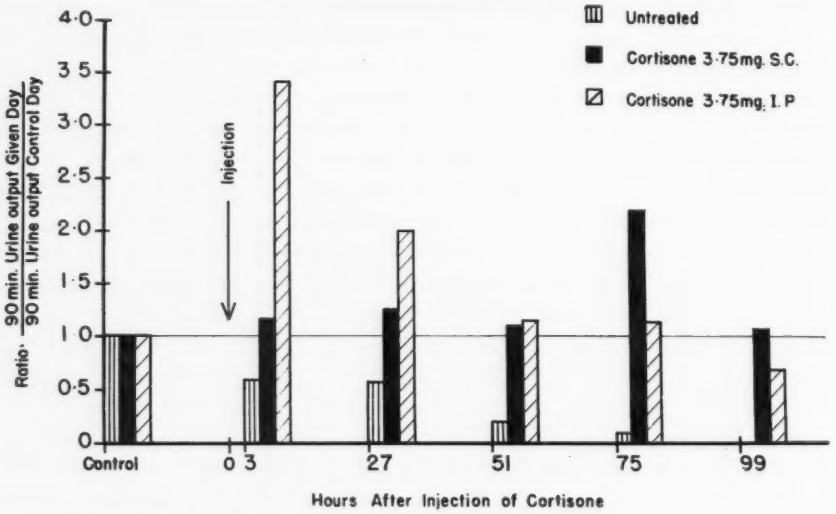


FIG. 5. The duration of effect of a single dose of cortisone, given intraperitoneally or subcutaneously, on the promotion of water diuresis in the adrenalectomized rat. (See text for details.)

four days. Cortisone s.c. had a slight effect for the first 3 days, but reached a maximum effect on day 4, after which the effect diminishes. Thus, there is no doubt that intraperitoneal cortisone has a definite and marked corticoid effect, as measured by this technique, and this effect is quicker, more intense, and of shorter duration than that produced by the same dose of cortisone given s.c. This is all the more remarkable since other corticoid effects such as growth inhibition, thymic atrophy and adrenal atrophy, as shown above, were not produced by intraperitoneally injected cortisone.

It would seem that in order to obtain maximum metabolic effects of crystalline cortisone acetate, a continuous high level must be maintained, such as might be expected from the continuous absorption of steroid from a subcutaneous depot. On the other hand, when intermittent high levels are induced, as with cortisone injected intraperitoneally, individual metabolic effects may be noted, such as the restoration of water diuresis in the adrenalectomized animal, but other effects, such as growth inhibition and adrenal atrophy may be absent.

SUMMARY

Cortisone acetate was administered intraperitoneally and subcutaneously to adult male and female rats in doses of 1.25 to 3.75 mg. daily for periods of 40 to 48 days. Subcutaneously administered cortisone acetate

produced the expected body weight loss, and atrophy of the thymus and adrenal cortex. The same dosage of cortisone acetate intraperitoneally did not cause weight loss, and produced no adrenal or thymic atrophy. The duration of effect of a single dose of cortisone was then studied by following the urine output of adrenalectomized rats subjected to a daily water load. It was found that intraperitoneal cortisone acetate had an immediate and rapid effect on the restoration of water diuresis in the water-loaded adrenalectomized rat, whereas subcutaneous cortisone acetate had a slower and more prolonged effect. The data suggest that certain effects of cortisone acetate, such as loss in body weight and atrophy of the thymus and adrenal cortex, may require a sustained corticoid level, as may be produced by subcutaneous administration of the drug. Other metabolic effects, such as the restoration of water diuresis in the adrenalectomized water-loaded rat, occur rapidly following intraperitoneal administration of cortisone acetate, but, perhaps because of the shorter duration of the effective corticoid level, there is no inhibition of growth or production of adrenocortical atrophy.

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THE EFFECT OF PROLONGED LOCAL TREATMENT WITH ADRENOCORTICAL AND SEX STEROIDS ON THE INTRADERMAL SPREADING ACTION OF HYALURONIDASE[1,2]

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THE inhibition of hyaluronidase activity as it affects the dermal spreading of an indicator substance by an acutely elevated blood level of either endogenously or exogenously supplied adrenocortical steroids (Opsahl, 1949 a,b,c; Winter and Flataker, 1950; Seifter, Baeder and Begany, 1949; Seifter *et al.*, 1950; Seifter, Baeder and Dervinis, 1949) has been confirmed (Hayes and Baker, 1951). Prolonged local application of whole adrenocortical extract, however, produced an accentuation in the activity of and an alteration in the pattern of spreading by the enzyme hyaluronidase (Hayes, Reed and Baker, 1950) when changes in area of spreading are measured as a function of time (Hayes and Reed, 1950). The change in enzyme activity was limited to the exact area of treatment and no systemic effect could be detected by this test. Employing pure crystalline steroids, Hayes and Bridgman (1951) reported that the prolonged local application of 11-dehydro-17-hydroxycorticosterone and desoxycorticosterone duplicated the results obtained with the prolonged local application of whole adrenocortical extract (Hayes, Reed and Baker, 1950). Progesterone, on the other hand, produced an accentuated spreading effect but with a different pattern of activity, quite unlike that obtained with adrenocortical extract or the adrenocortical steroids (Hayes and Bridgman, 1951). This report deals with an extension of these studies as related to specific pure crystalline steroids.

MATERIALS AND METHODS

The free alcoholic steroids selected for investigation were 17-hydroxycorticosterone (compound F),[4] corticosterone (compound B),[4] estradiol[5] and testosterone.[5] These sub-

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[2] The technical assistance of Mrs. A. D. Bridgman is gratefully acknowledged.

[3] Present address: Department of Surgery, Yale University School of Medicine, New Haven, Connecticut.

[4] The supplies of free alcoholic compounds F and B and the hyaluronidase (Alidase) employed in this study were generously supplied by Dr. Irwin C. Winter, of G. D. Searle Company, Chicago, Illinois.

stances were dissolved in absolute ethyl alcohol at a concentration of 1 mg./cc.

Thirty-six litter mate male rats of the Long-Evans strain in the initial weight range of 200 ± 50 gm. were used, kept in individual cages and fed a standard laboratory diet *ad libitum*. There were 6 animals in each of the 4 experimental groups; one group for each hormone. For 6 months 0.1 cc. of each of the steroid solutions was applied daily to the dorsal cervical region just caudal to the right ear of each of 6 animals. Twelve control animals were treated similarly but with only the solvent, absolute alcohol, in the same manner. The area of treatment in all animals was approximately 10 sq. cm. In all 5 groups (control plus experimental) the left side of each animal was left untreated. Body weights were determined and regrown hair on the neck was clipped at weekly intervals after hair growth patterns had been recorded (Butcher, 1934). The technic employed for study of the spreading action of hyaluronidase has been described in detail by Hayes and Reed (1950). The hair was clipped closely from the proposed injection sites on the dorsum of the neck and the animals were secured loosely for constancy of position. The animals were quieted by the subcutaneous injection of 0.3 cc. of a veterinary solution of sodium pentobarbital (6.5% solution) and were anesthetized lightly with ether only for the duration of the initial injection. The indicator employed was an isotonic solution of hemoblogin. The final injection quantity was 0.05 cc. containing 25 units of hyaluronidase.[4] The rate of injection was 0.01 cc./5 sec.

The manipulations of the injection procedure were controlled carefully and the *intradermal* injections were made in comparable sites on each side of the dorsal cervical midline, that is on the treated and untreated side of each animal.

Measurements were made of the length and width of the ellipsoidal area of spread. The first measurement, which was made at the termination of the injection, was taken as a baseline. Then, observations were made every minute for 15 minutes; every 5 minutes through 30 minutes; every 10 minutes through one hour and every 15 minutes through 2 hours. The increase in area for each time interval over that of the initial injection area was calculated, the arithmetic means were plotted using the ordinate as the increase in area of spread over the initial area in sq. mm. and the abscissa as the logarithm of the time in seconds.

OBSERVATIONS

Though no attempt at controlling food intake was made, it is significant that the control group mean weight gain over the period of study was 189 gm.; the compound F group mean weight gain was 184 gm.; the compound B group mean weight gain was 181 gm.; the testosterone group mean weight gain was 186 gm.; but, the estrogen group mean weight gain was only 18 gm. Of all the observations of spreading activity measured, 87% fell within a range of 4 sq. mm. of the plotted mean. There was no significant difference between observations made on the untreated and on the vehicle treated sides of the control animals. The results of the control tests are graphed as the X curve in all the accompanying illustrations. The effect of prolonged local treatment with testosterone and corticosterone

[5] The estradiol and testosterone in free alcoholic form were supplied generously by Ciba Pharmaceutical Products of Summit, New Jersey.

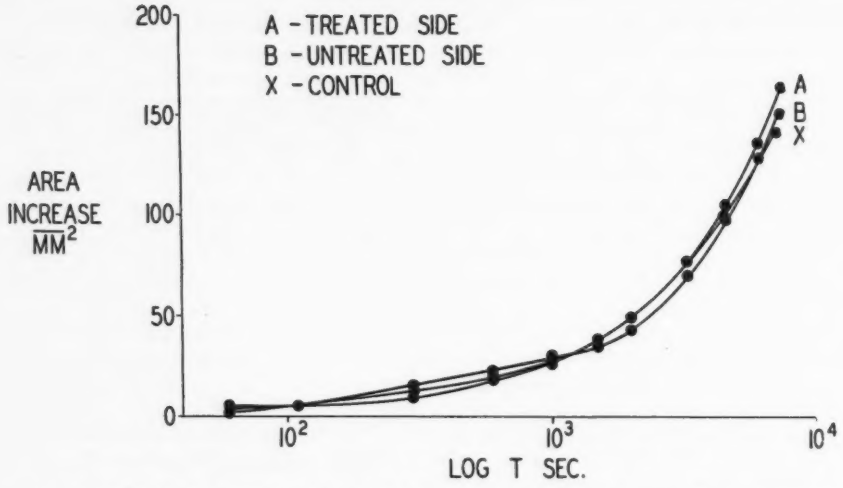

FIG. 1. Effects of the Chronic Local Administration of Testosterone. X is the control (see text). There is no significant difference between the treated and the untreated sides or either side and the controls.

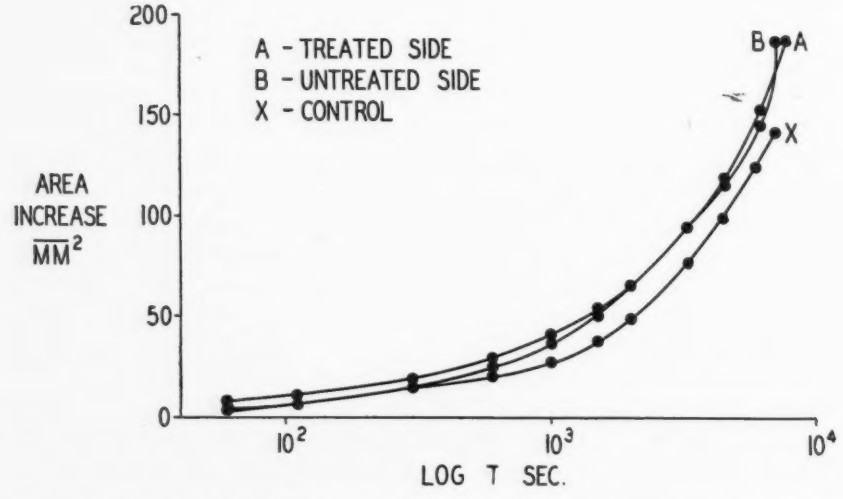

FIG. 2. Effects of the Chronic Local Administration of Corticosterone. X is the control (see text). There is no significant difference between the treated and the untreated sides or either side and the control.

on the usual pattern[6] of hyaluronidase intradermal spreading of a hemoglobin solution is well demonstrated in Figures 1 and 2. There is no significant alteration in either the pattern or extent of the spreading action. Figure 3 demonstrates the alteration in spreading caused by the prolonged

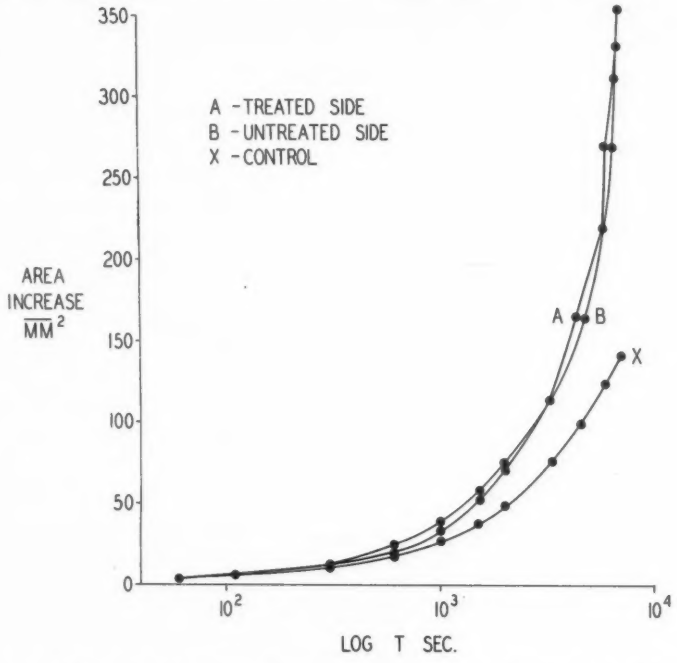

FIG. 3. Effects of the Chronic Local Administration of Estrogen. X is the control (see text). There is no alteration in the pattern of spreading but there is a very marked accentuation in rate of spreading in the rapid phase. There is no significant difference between treated and untreated sides, indicating a profound systemic effect.

local administration of estrogen. That there is a potent systemic effect is apparent since there appears to be no difference between the treated and untreated sides. In addition, the principal effect appears to be on the rapid phase of spreading and is comparable to the effect after similar treatment with progesterone (Hayes and Bridgman, 1951).

In sharp contrast are the results obtained after the prolonged local application of 17-hydroxycorticosterone (Fig. 4). The slow phase of spreading is almost completely abolished and there is increased rate of spreading in the rapid phase.

[6] All spreading activity is composed of two phases: an early slow phase followed by a late or rapid phase. Such a sequence is named pattern of spreading.

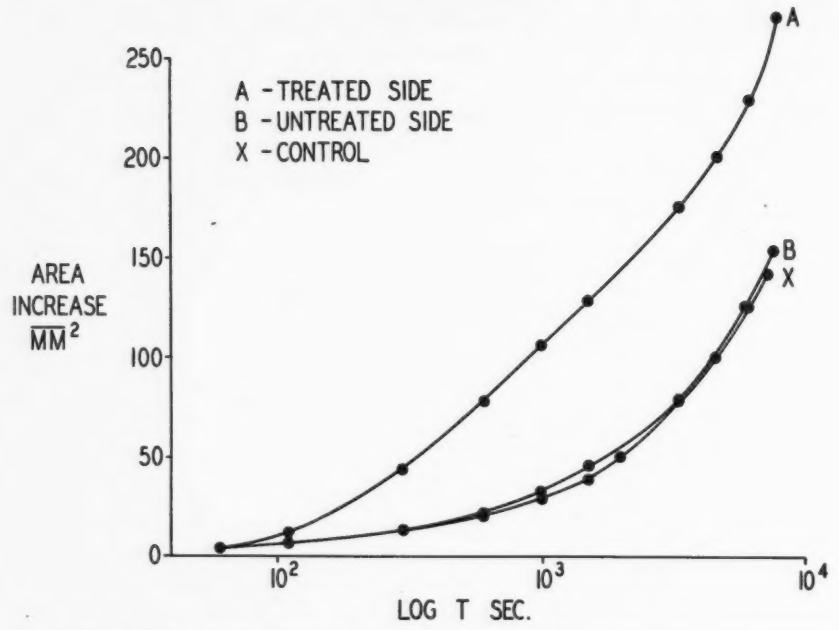

FIG. 4. Effects of the Chronic Local Administration of 17-hydroxycorticosterone. X is the control (see text). There is no significant difference between the control and the untreated side of the experimental animals. The treated side demonstrates an accentuation of spreading activity and a definite alteration in pattern of spreading, characterized principally by a change in the slow phase of spreading.

DISCUSSION

In this investigation prolonged local treatment with corticosterone or testosterone produced no alteration in the extent, rate or pattern of spreading. Prolonged estrogen local treatment resulted in a marked systemic response evidenced by growth inhibition and a striking alteration in rate of spreading; it is significant that there was no great alteration in the pattern of spreading. The use of 17-hydroxycorticosterone resulted in an alteration of rate and of pattern of spreading which has been reported to be characteristic with whole adrenocortical extract, whether applied locally for a prolonged period (Hayes, Reed and Baker, 1950) or given systemically in a chronic experiment (Hayes and Baker, 1951).

When the spread of an indicator without enzyme was studied in locally pretreated skin, no essential change in the manner or extent of spreading activity was observed (Hayes, Reed and Baker, 1950). This finding would exclude the thinness of the skin which exists under these conditions (Baker and Whitaker, 1948; Castor and Baker, 1950) as being the cause

of the accelerated rate of spread. It is only reasonable to conclude, then, that such prolonged treatment with adrenocortical steroids alters the substrate, ground substance, in an unknown fashion rendering it more susceptible to enzymatic hydrolysis. It is of considerable significance that the ability to alter the slow phase of spreading appears to be a characteristic of only certain steroid hormones with an adrenocortical origin.

SUMMARY

Prolonged local application of corticosterone or testosterone does not influence the spreading action of hyaluronidase. Similar treatment with estrogen produces a profound systemic effect and accentuates hyaluronidase spreading, but only on the rapid phase. On the contrary, 17-hydroxycorticosterone produces an effect restricted to the area of treatment characteristic of adrenocortical steroids: accentuated rate of spreading and nearly complete abolition of the slow phase.

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THE EFFECT OF HYPOPHYSECTOMY ON THE REGENERATION OF THE LIVER AFTER PARTIAL HEPATECTOMY IN DOGS

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REGENERATION of the remaining portion of the liver after partial hepatectomy in the dog is rapid and complete within five to eight weeks (Mann, 1927; Fishback, 1929; Bollman and Mann, 1936; Grindlay and Bollman, 1952). Hypophysectomy was found to have little effect on regeneration of the liver in the albino rat if such factors as body growth and intake of food were considered. Franseen and co-workers (1938) and Higgins and Anderson (1931) and Higgins and Ingle (1939) came to the same conclusion, namely, that the retarded restoration of the remaining portion of the liver after the removal of the hypophysial body was largely if not entirely due to reduction in the amount of food consumed by the hypophysectomized rat. Canzanelli and associates (1949), in a more recent study, found great diminution in regeneration and content of nucleic acid of the liver after partial hepatectomy and hypophysectomy.

Because data on the effect of hypophysectomy on regeneration of liver in dogs were not found in the literature and because hypophysectomy affects very little the amount of food consumed by the dog, it was deemed desirable to study regeneration of the liver in dogs after hypophysectomy.

METHODS

Six adult dogs, apparently healthy and weighing from 7 to 12 kg., were used.

The pituitary body was removed by the transbuccal approach according to a modification of McLean's procedure (McLean, 1928; Essex and Astarabadi, 1952). The operation was considered successful when tissue characteristic of adenohypophysis (pars distalis) was not seen after thorough examination of a series of sections taken from the tissue remnant in the sella turcica at the end of the experiment. According to the criteria used only 3 dogs of the series of 6 were found to have had complete hypophysectomies.

Partial hepatectomy was carried out according to the method published elsewhere (Grindlay and Bollman, 1952). By this method about 70% of the liver was removed. Partial hepatectomy was done ten to twelve days after removal of the hypophysis cerebri.

The resected portion of the liver was weighed after the blood had drained out.

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Dextrose (50 gm. in 100 cc. of physiologic saline solution) was administered intravenously immediately before and 2 to 4 hours after partial hepatectomy. This was repeated twice daily until the dog resumed eating. It was found necessary to do this in order to prevent fatal hypoglycemia in the successfully hypophysectomized dogs.

The experiment was terminated by anesthetizing the dog, removing the remaining portion of the liver and weighing it in the same way as the portion removed at operation.

The amount of restoration was estimated by subtracting the calculated weight of the original remnant (which is 30% of the calculated weight of the whole liver or $\frac{3}{7}$ of the portion removed at operation) from the weight of the portion of the liver which remained at necropsy. To express the amount of restoration in per cent, the result of the subtraction was divided by the weight of the original remnant and multiplied by 100.

RESULTS

A significant difference was not found between the restored livers of the nonhypophysectomized dogs and those of the dogs which had a part or all of their adenohypophyses removed (Table 1).

TABLE 1. HEPATIC RESTORATION AFTER HYPOPHYSECTOMY AND PARTIAL HEPATECTOMY

Number and sex	Body weight		Liver weight, gm.		Per cent restoration	Days after hepatectomy	Conditions of adenohypophysis*
	Initial	Final	Removed	Final			
1 F	9.5	8.5	178	145	90	36	Absent
2 M	7	7.5	148	130	106	36	Absent
3 M†	12	11.5	140	200	233	47	Absent
Mean	9.5	9.2	152	146	143	39	
4 M	10	8.5	227	240	150	47	Partially regenerated
5 M	10.5	11	227	290	202	50	Intact
6 M	12	12	157	250	243	50	Regenerated
Mean	10.8	10.5	203	260	198	49	

* As revealed by histologic study of a series of sections from the tissue "remnant."
† Castrated.

Grossly, the restored livers looked the same in the two groups of animals. They were thick with rounded edges, showing conspicuous reticulation and firm consistency. The color of the livers, however, in the successfully hypophysectomized dogs was bright red, resembling the color of arterial blood or blood which had been saturated with oxygen, in contrast to the dark-red livers of the control dogs.

Histologic preparations of livers of control and hypophysectomized animals did not show any differences. The sections were cut ten microns in thickness and stained with hematoxylin and eosin.

The pancreas was decidedly pink in the hypophysectomized dogs as compared to the creamy color of the normal pancreas. This pinkish hue of the pancreas was reported by Sweet and Allen (1913) in hypophysecto-

mized dogs and by Mann and Drips (1915) in adrenalectomized dogs.

It has been found that hypophysectomized dogs do not tolerate well preoperative fasting for 24 to 48 hours. A number of animals under investigation died in hypoglycemic coma when fasted for 24 hours. Whether fasting *per se* or the combination of fasting and the removal of 70 per cent of the liver was responsible for the fatal hypoglycemia, remains undetermined. Values for blood sugar as low as 15 mg. per 100 cc. of blood have been found in hypophysectomized dogs fasted for 24 to 48 hours.

COMMENT

The data obtained from the successfully hypophysectomized dogs indicated that the remaining portion of the liver may be regenerated in about 40 days to more than 140% of the calculated control weight in response to the removal of 70% of its mass. In other words, the 30% of liver remaining after partial hepatectomy will grow and after 40 days will weigh as much as the original liver. These findings are in accord with the work done on rats, in which forced feeding of hypophysectomized rats resulted in restoring the weight of the liver to normal both with and without partial hepatectomy (Franseen and co-workers, 1938; Levin, 1944; McQueen-Williams and Thompson 1939–1940). Therefore the reduction in the weight of the liver which follows hypophysectomy is secondary to the decreased amount of food that is consumed by the hypophysectomized rat and is not caused primarily by the absence of a "specific splanchnotrophic" hormone of the pituitary body. That such a specific interdependence does not exist between the pituitary body and the liver in the dog is definitely shown by the results of this study.

The hypoglycemia which caused the death of many of the hypophysectomized dogs at the beginning of the investigation deserves comment. In a normal dog, removal of 70% of the liver is usually followed by rapid and uneventful recovery (Grindlay and Bollman, 1952). On the other hand, a hypophysectomized dog after removal of 70% of the liver has been found to manifest severe symptoms of hypoglycemia, namely, profuse salivation, weakness, generalized muscular twitching, and finally, if sufficient glucose is not given, convulsions, coma and death. The striking similarity of the symptoms just described to those of dogs after complete hepatectomy is recognized. In this respect hypophysectomy can be considered to have a decidedly adverse effect on the concentration of glucose in the blood after partial hepatectomy. Fasting for as brief a period as 24 hours likewise may, in certain instances, reduce the concentration of glucose in the blood of the hypophysectomized animal to a level incompatible with life. It is not surprising that the hypoglycemia of the hypophysectomized dog has been found to be greatly aggravated by partial hepatectomy.

It is possible that in the absence of the hypophysis cerebri, the change

of the glycogen in the liver to the glucose in the blood is held in abeyance or is abolished altogether or the amount of glycogen which is contained in the residual portion of the liver after partial hepatectomy may not be enough to maintain the normal level of glucose in the blood. It is highly probable that in the absence of the hypophysis, the glucose of the blood is chiefly derived from the carbohydrate of the diet rather than from hepatic stores of glycogen. Severe hypoglycemia was not seen after partial hepatectomy if the dog had resumed eating. If the animals did not eat the food, glucose had to be given parenterally, or a fatal outcome was inevitable.

An adequate explanation for the intriguing colors of both the liver and pancreas of the hypophysectomized dogs cannot be given at present, but it seems questionable that they are produced entirely by changes in the state of the blood vessels of these organs.

SUMMARY AND CONCLUSIONS

The effect of hypophysectomy on regeneration of the liver after partial hepatectomy was studied in dogs, and it has been found that the absence of the hypophysis cerebri does not seem to interfere with regeneration of the liver.

Severe and sometimes fatal hypoglycemia developed in a few of the completely hypophysectomized dogs after fasting. It was hastened and aggravated by partial removal of the liver.

Changes in the color of the liver and pancreas have been noticed in hypophysectomized dogs.

Acknowledgment

The technical assistance of Mr. W. A. Meeker is acknowledged with thanks.

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STUDIES ON THE RATES OF INVOLUTION AND RECONSTITUTION OF LYMPHATIC TISSUE[1]

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MARKED lymphatic tissue involution has been demonstrated under conditions of inanition, irradiation and chronic treatment with adrenocortical hormones. Starvation provides certain unique advantages for the investigation of adrenocortically mediated acute lymphatic tissue involution. It is now known that: (1) loss of lymphatic tissue weight during a period of inanition begins earlier and proceeds at a more rapid rate than that of other body tissues (Jolly, 1914) and, (2) the rate of release of nitrogen from lymphatic tissue in the starved animal is under adrenocortical hormone control (White and Dougherty, 1947).

Although there is an extensive amount of literature on the involution of lymphatic tissue produced by inanition, little is known concerning its reconstitution. A series of experiments was designed to compare the influences of fasting and refeeding on lymphatic tissue of intact and adrenalectomized mice.

MATERIALS AND METHODS

A total of 515 CBA mice of both sexes between 12 and 16 weeks of age was used in these experiments. Animals were kept at constant temperatures and segregated according to sex. All mice that were fed were given dog chow (Purina) and water *ad libitum*. Fasted mice were kept in individual containers with screen bottoms to prevent coprophagy.

The control group consisted of 150 intact-fed mice. The experimental animals were divided into fasted and refed groups. The fasted animals studied were: (1) Intact mice fasted; different groups were killed at 24, 48, 72 and 96 hours. (2) Intact mice fasted 48 hours, then adrenalectomized and fasting continued. Groups of these starved, adrenalectomized animals were killed at 6, 12, 24 and 48 hours after operation.

Refed animals included: (1) Intact mice fasted 96 hours and refed for a period of 16 days, during which time groups of animals were killed on successive days. (2) Intact mice fasted 96 hours, adrenalectomized and refed for a period of 8 days. Groups of animals were killed at refeeding intervals of 2 days.

All animals were weighed at the beginning of the experiment and at the time of kill.

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TABLE 1. MG. LYMPHATIC TISSUE PER 100 GMS. FINAL BODY WEIGHT OF INTACT AND ADRENALECTOMIZED-FASTED MICE

Hours of fast	Intact fasted	Fasted adrenect*	Intact fasted	Fasted adrenect*
	Total lymphatic organ sample		*Thymus*	
0	356 ±11†		172 ± 7.5	
24	329.6±22		166.9±10.1	
48	174.3±14.5		72.0± 9.6	
54	—	258.0±14.0	—	120.0±10.8
60	—	218.6±23.8	—	89.3±12.4
72	178.0±10	226.0±14.9	63.2±6.8	90.0± 8.8
96	134 ±12	184.0±12.6	31.2±2.3	59.2± 5.4
	Spleen		*Mesenteric node*	
0	330 ±14.6		128 ±10	
24	231.9±16.7		119.8±11.4	
48	160.0± 8.9		68.5± 3.6	
54	—	218.0± 9.9	—	102.0± 9.0
60	—	193.0± 7.2	—	95.8± 9.4
72	142.2±10.4	221.0±14.6	83.2±6.6	94.0± 6.0
96	112.7± 8.3	184.0±11.6	87.5±9.6	87.0± 6.2
	Inguinal nodes		*Axillary nodes*	
0	28.5± 2.8		27.5± 2.5	
24	21.3± 1.1		21.6± 1.3	
48	18.0± 2.1		15.8± 2.2	
54	—	18.8± 1.8	—	17.4± 1.4
60	—	17.7± 1.7	—	15.6± 1.4
72	15.4± 1.2	21.8± 2.3	15.2±1.3	19.9± 2.1
96	8.0± 0.6	18.5± 1.5	7.4±0.5	19.3± 1.5

* Adrenalectomized after 48 hours fast and continued to fast.
† 0, 24, and 48-hour weights for both intact and adrenalectomized groups.

The lymphatic organs examined were paired inguinal, axillary and cervical nodes, thymus, mesenteric node and spleen. These organs were dissected free of connective tissue and weighed by means of a torsion balance. Both the individual lymphatic organ weights and the weight of the pooled lymphatic organs were obtained for each animal. The weights of the aggregates of lymphatic organs do not include the spleen and are referred to as the total lymphatic organ sample. Nitrogen determinations, however, were performed on the pooled total lymphatic organ samples and included the spleens. Nitrogen content was determined by semi-micro Kjeldahl analysis and is expressed as mgs. of nitrogen/gm. of lymphatic tissue.

Bilateral adrenalectomy was performed under ether anesthesia by the usual dorsal approach. Tail blood was used for all leucocyte counts. Differential blood counts of 200 cells were done on films stained with May-Grunwald Giemsa. Lymphatic organs (spleens, mesenteric nodes, thymi) from intact-fed, intact-fasted, and fasted-adrenalectomized mice were fixed in Zenker's solution and stained with methyl-green pyronin for histological study. Mitotic indices[2] were calculated by counting the number of mitotic figures per thousand cells in areas of the same cellular density.

Organs weights of refed mice were expressed in terms of 100 gm. of body weight of control-intact-fed mice of the same age and sex as the experimental animals at time of kill. Organ weights of fasted animals were expressed in terms of 100 gm. of final body weight of the experimental animal.

[2] The authors wish to thank Mr. Gottlieb Schneebeli for his expert histological preparations and his aid in performing the mitotic indices.

RESULTS

Effect of fasting on lymphatic tissue of intact mice. It may be seen from Table 1 and Figure 1 that the weights of the total lymphatic tissue sample decreased rapidly during starvation. After a 96-hour fast the total lymphatic tissue sample had lost 63% of its initial weight as compared to a 32% loss of body weight. The maximum decrement occurred between 24

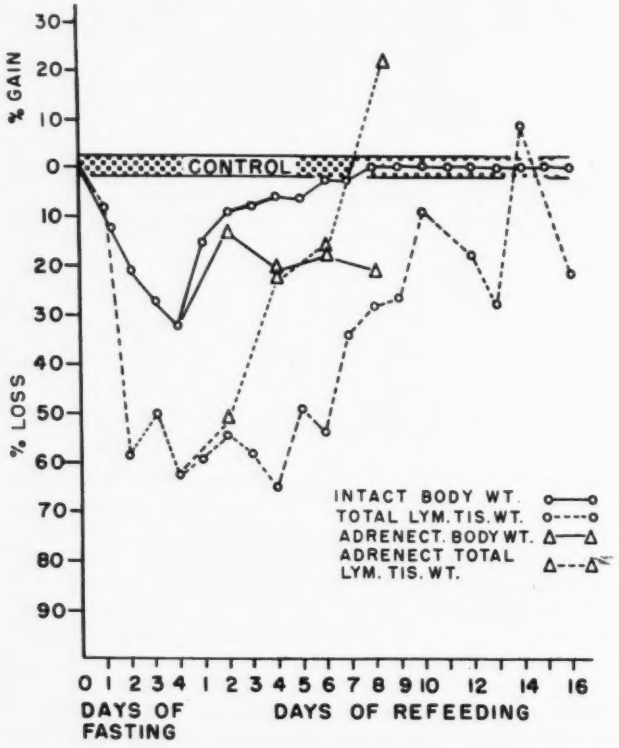

FIG. 1. Per cent weight changes of total lymphatic tissue weights and body weights of intact mice fasted 4 days and refed 16 days and of intact mice fasted 4 days, adrenalectomized and refed 8 days.

and 48 hours whereas the body lost weight at a fairly constant rate during the entire starvation period (Fig. 1).

The responses of individual lymphatic organs to fasting are recorded in Table 1. Individual lymphatic organs differed in degree and rate of weight loss. The thymus proved to be the most sensitive lymphatic organ to inanition. It lost 66% of its initial weight by 48 hours and 83% by the end of the fasting period (Fig. 2). The spleen lost 66% of its initial weight by

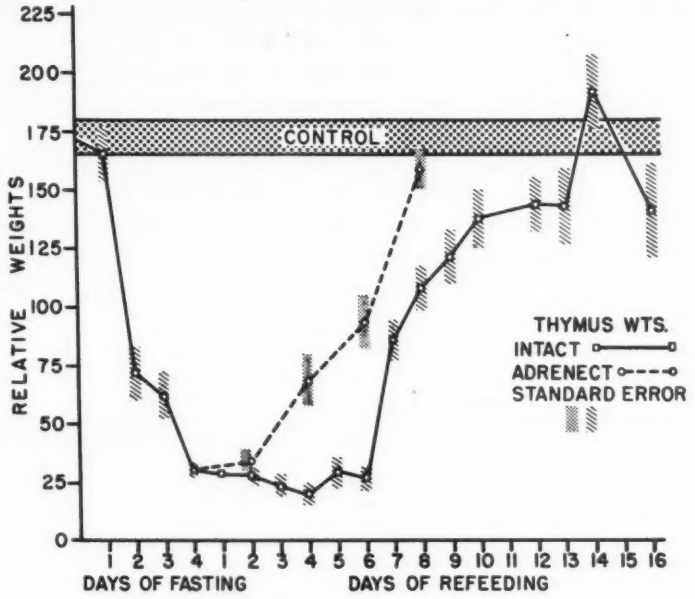

FIG. 2. Relative thymus weights of intact mice fasted 4 days and refed 16 days and of intact mice fasted 4 days, adrenalectomized and refed 8 days. Fasting weights expressed in mg. lymphatic tissue/100 gm. final body weight of experimental animal. Refed weights expressed in mg. lymphatic tissue/100 gm. body weight of intact-fed mice of the same age and sex as experimental mice.

TABLE 2. INFLUENCE OF FASTING, ADRENALECTOMY AND REFEEDING ON ABSOLUTE NUMBERS OF LEUCOCYTES

Groups	Number of animals	Time of fasting or refeeding	W.B.C., thousands per cmm.	Lymphocytes, thousands per cmm.	Polymorphonuclears, thousands per cmm.	Eosinophils per cmm.
			Mean S.E.	Mean S.E.	Mean S.E.	Mean S.E.
Intact controls	150	—	12.6±0.3	8.6±0.3	3.9±0.1	117± 8.0
Intact fasted	10	24 hrs.	16.7±1.9	8.5±1.2	8.1±2.5	101± 37.0
	19	48 hrs.	13.9±0.9	5.4±1.0	8.6±0.8	34± 14.0
	10	72 hrs.	10.6±1.1	3.1±0.4	7.6±1.2	0±
	28	96 hrs.	11.3±1.0	5.2±0.7	6.1±0.6	28± 11
Adrenalectomized fasted*	14	54 hrs.	21.5±1.9	14.4±4.2	6.2±0.9	299± 69
	9	60 hrs.	23.5±2.5	19.6±2.1	4.0±0.8	15± 15
	9	72 hrs.	17.1±1.9	14.8±2.3	2.3±0.4	28± 19
	13	96 hrs.	24.1±7.1	17.1±3.6	7.0±1.2	43± 29
Intact refed†	10	1 day	13.0±1.4	5.8±1.3	7.1±1.1	50± 40
	10	2 days	11.4±0.8	4.4±0.7	6.9±0.9	30± 20
	10	4 days	14.8±1.4	10.5±1.1	4.3±0.5	20± 20
	12	8 days	20.6±1.1	14.3±1.3	6.1±1.3	150± 45
	10	12 days	12.4±1.3	8.0±0.8	4.3±1.2	80± 45
	5	16 days	20.1±2.7	11.9±1.0	4.9±0.9	200± 25
Adrenalectomized refed‡	13	2 days	15.8±2.5	11.9±0.6	3.8±0.5	79± 34
	9	4 days	16.0±2.5	10.5±1.4	4.9±0.6	104± 37
	8	6 days	20.3±2.2	11.6±1.1	8.3±1.2	463±119
	6	8 days	18.7±2.2	12.7±2.2	4.5±0.6	1285±438

* Adrenalectomized following a 48-hour fast and continued to fast.
† Previously fasted 96 hours.
‡ Adrenalectomized following a 96-hour fast.

96 hours (Fig. 3). The mesenteric node lost 48% of its weight by 48 hours but then gained so that by 96 hours only a 31% weight loss was evident (Fig. 4). Inguinal and axillary nodes began to lose weight during the second day and had lost 64% of their initial weight by the end of the fasting period (Fig. 5).

The ratio of nitrogen content per unit weight of lymphatic tissue remained constant during the entire fasting period (Fig. 6).

There was no significant change in the total leucocyte count during fasting. A polymorphonuclear leucocytosis was found at all time inter-

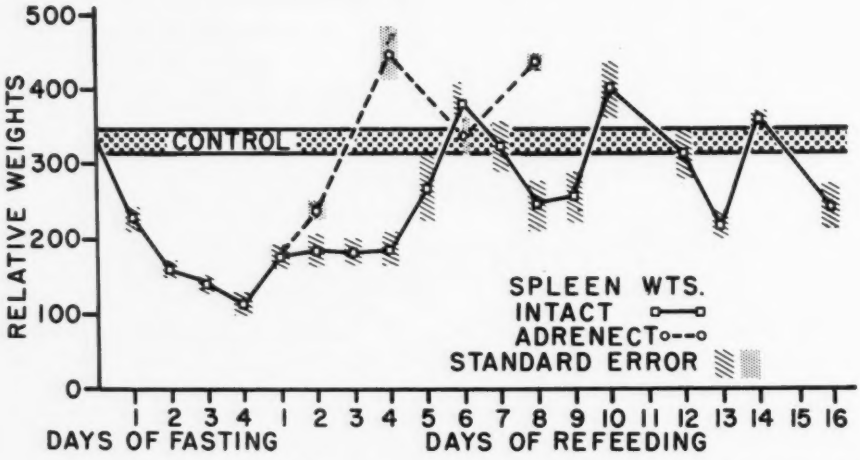

FIG. 3. Relative spleen weights of intact mice fasted 4 days and refed 16 days and of intact mice fasted 4 days, adrenalectomized and refed 8 days. Fasting weights expressed in mg. lymphatic tissue/100 gm. final body weight of experimental animal. Refed weights expressed in mg. lymphatic tissue/100 gm. body weight of intact-fed mice of the same age and sex as experimental mice.

vals. A lymphopenia and eosinopenia occurred by 48 hours of fast and persisted throughout the fasting period (Fig. 7, Table 2).

Effect of adrenalectomy on lymphatic tissue of fasted mice. As seen in Table 1 and Figure 8, adrenalectomy after 48 hours of fast arrested the involution of lymphatic tissue. In fact, 6 hours after operation an actual increase in lymphatic tissue weight was observed. Although 12 hours after operation (72 hours of fast) the total lymphatic tissue again began to lose weight, the loss of weight was still not as great as that of the intact animals by the end of the fasting period. Individual lymphatic organ weights were significanlty higher than those of the intact group at the end of the fasting period with the exception of the mesenteric node (Table 1).

A marked lymphocytosis (as compared to normal levels found in intact-

TABLE 3. NUMBER OF MITOTIC FIGURES PER 1000 CELLS (MITOTIC INDEX) IN MALPIGHIAN CORPUSCLES, THYMIC CORTICES AND GERMINAL CENTERS OF LYMPHATIC ORGANS IN FED, FASTED AND FASTED-ADRENALECTOMIZED MICE

Groups	Number of mice	Hours of fast	Mitotic index*		
			Spleen	Thymus	Mesenteric node
Intact fed	5	—	12.0	15.6	12.0
Intact fasted	5	48	5.2	0.4	8.0
	5	96	5.5	0.2	4.3
Adrenalectomized at 48 hours of 96-hour fast	5	54	3.0	3.0	10.6
	5	60	11.2	1.0	15.0
	5	72	16.0	3.6	15.4
	5	96	27.6	15.6	29.7

* Mean values for group (5000 cells/mouse).

fed animals) was observed 6 hours after adrenalectomy and was at a higher level at 12 hours (Fig. 9). At 24 hours (72 hours of fast) the circulating lymphocytes had decreased to the lymphocytotic level characteris-

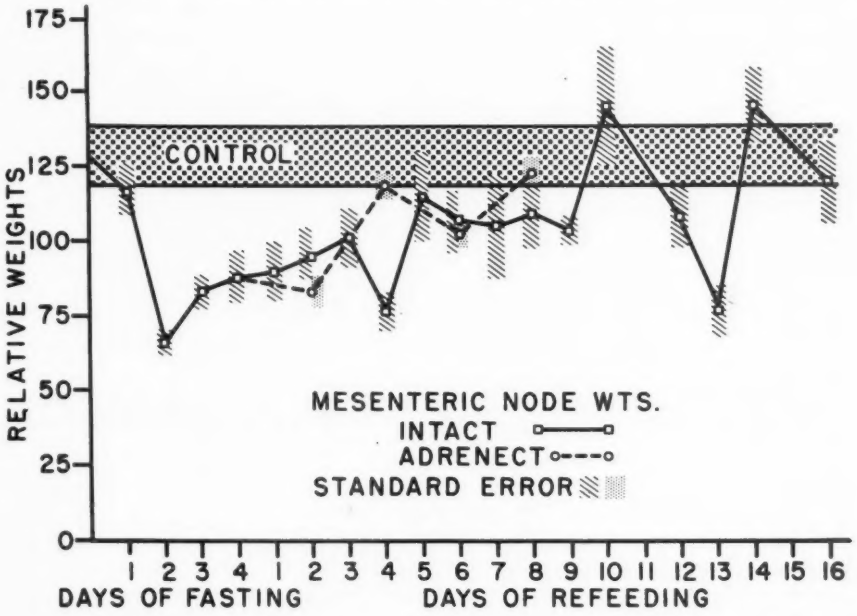

FIG. 4. Relative mesenteric node weights of intact mice fasted 4 days and refed 16 days and of intact mice fasted 4 days, adrenalectomized and refed 8 days. Fasting weights expressed in mg. lymphatic tissue/100 gm. final body weight of experimental animal. Refed weights expressed in mg. lymphatic tissue/100 gm. body weight of intact-fed mice of the same age and sex as experimental mice.

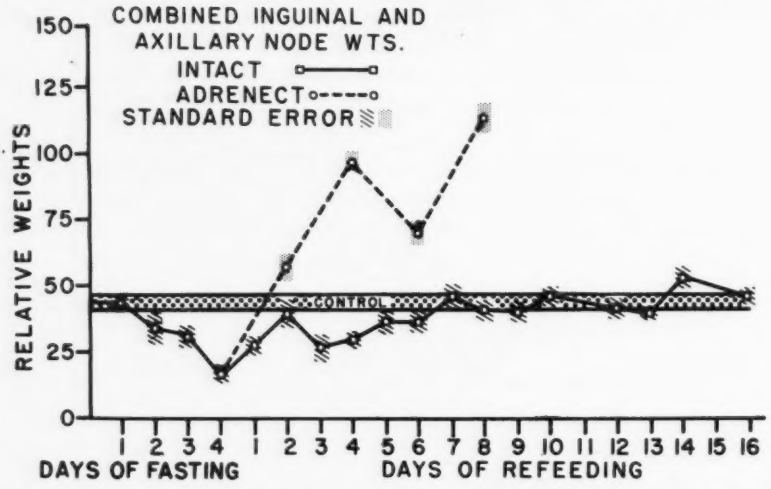

FIG. 5. Relative combined inguinal and axillary node weights of intact mice fasted 4 days and refed 16 days and of intact mice fasted 4 days, adrenalectomized and refed 8 days. Fasting weights expressed in mg. lymphatic tissue/100 gm. final body weight of experimental animal. Refed weights expressed in mg. lymphatic tissue/100 gm. body weight of intact-fed mice of the same age and sex as experimental mice.

tically seen in adrenalectomized-fed mice and remained at this level throughout the fasting period.

Effect of fasting and adrenalectomy on mitotic indices of lymphatic tissue.

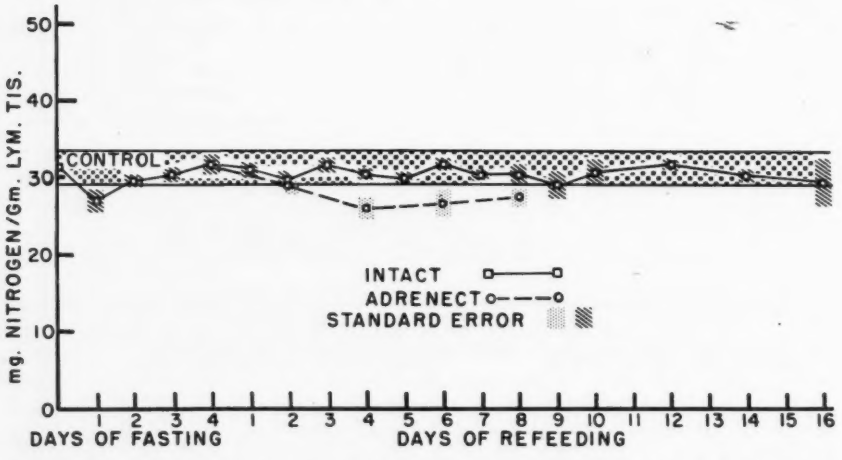

FIG. 6. Mg. nitrogen/gm. lymphatic tissue of intact mice fasted 4 days and refed 16 days and of intact mice fasted 4 days, adrenalectomized and refed 8 days.

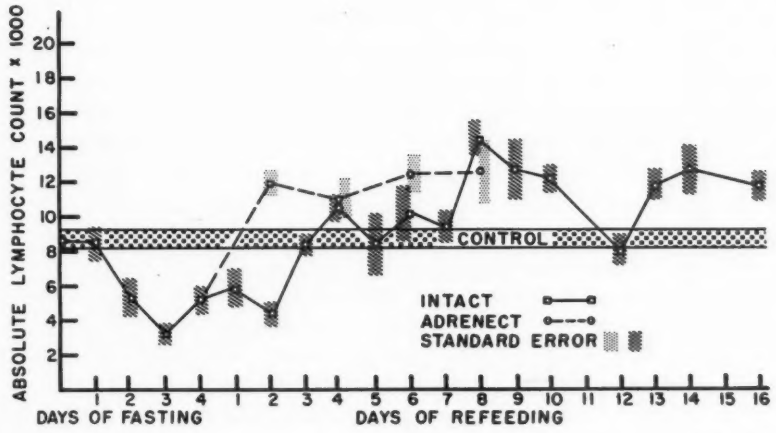

FIG. 7. Absolute lymphocyte counts of intact mice fasted 4 days and refed 16 days and of intact mice fasted 4 days, adrenalectomized and refed 8 days.

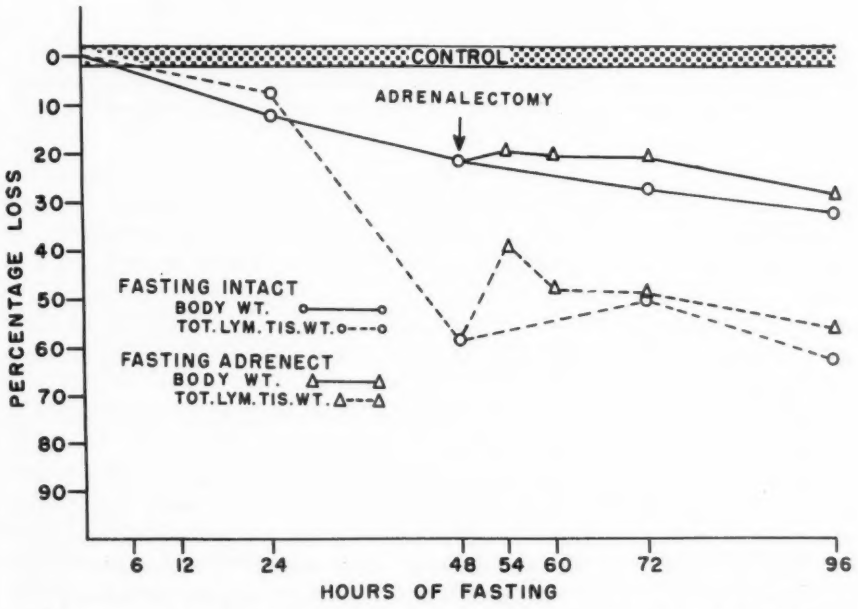

FIG. 8. Per cent loss of total lymphatic tissue weight and body weight of intact mice fasted 96 hours and of intact mice fasted 48 hours, adrenalectomized and continued to fast to 96 hours.

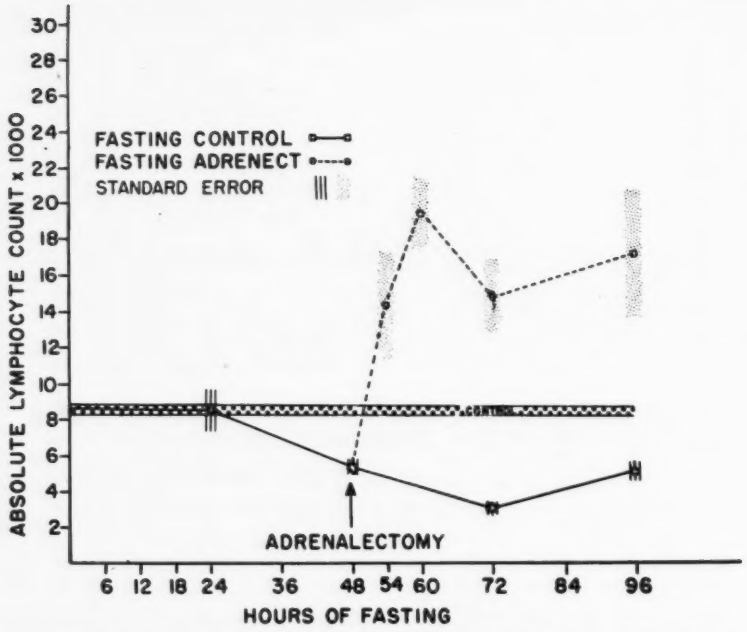

FIG. 9. Absolute lymphocyte counts of intact mice fasted 96 hours and of intact mice fasted 48 hours, adrenalectomized and continued to fast to 96 hours.

The number of mitotic figures in all lymphatic organs studied decreased during starvation. The thymus was outstanding in this respect; the mitotic index decreased from a control value of 15.6 to 0.4 at 48 hours and to 0.18 at 96 hours. When mice were adrenalectomized following a 48-hour fast and fasting was continued, a marked increase of the number of cells undergoing mitosis was found. The mitotic index of the thymus did not increase until 96 hours and then to a lesser degree than that of the spleen and mesenteric node (Table 3).

Effect of refeeding on lymphatic tissues of previously fasted intact and adrenalectomized mice. Body weights of previously fasted-intact mice increased during the first day and reached the weight range of fed animals of the same age by the third day (Fig. 1). On the other hand, the weight of the total lymphatic tissue sample did not begin to increase significantly until the fifth day of refeeding. A rapid increase in weight was then found so that by the tenth day the total lymphatic tissue weight was considered to be within the range of the total lymphatic tissue weight of intact-fed mice of the same age (Fig. 1).

Individual lymphatic organs differed in response to refeeding. Thymi of

intact animals did not begin reconstitution until the sixth day of refeeding at which time a rapid gain in weight was found which attained the level characteristic of fed mice of the same age by the tenth day (Fig. 2). The spleen responded earlier and was found to return to its control range by the fifth day (Fig. 3). Although the weights of mesenteric nodes fluctuated more than those of other lymphatic organs during the entire refeeding period, weights within the range for fed-intact mice were attained by the fifth day (Fig. 4). The combined inguinal and axillary node weights were found to return rapidly to normal values by the second day of refeeding (Fig. 5).

In contrast to the intact mice which regained their body weight by the third day of refeeding, the body weight of the refed-adrenalectomized group did not return to pre-fasting levels by the end of the 8-day period of study (Fig. 1). However, the total lymphatic tissue weight was found to be increased the second day and approached normal values by approximately the sixth day (Fig. 1). In addition, in the adrenalectomized animals, no individual lymphatic organ differences were found with respect to rate of reconstitution following refeeding (Figs. 2, 3, 4, 5). The gain in total lymphatic tissue weight began almost immediately upon refeeding and continued to increase in a linear fashion. Thus, the complete reconstitution of the weight of lymphatic tissue of these animals occurred much sooner than did that of intact-refed mice (Fig. 1).

The nitrogen content per unit of lymphatic tissue weight remained the same in both intact and adrenalectomized mice during the refeeding period (Fig. 6).

Absolute numbers of lymphocytes of intact mice were found to be within the normal range for intact-fed animals by the third day of refeeding. However, in adrenalectomized mice an absolute lymphocytosis (attained within 48 hours) was noted throughout the refeeding period (Table 2 and Fig. 7). Absolute numbers of eosinophils of intact mice remained at low levels until the fourth day of refeeding; the eosinophils of adrenalectomized mice increased from a slight eosinopenia (79/cmm.) on the second day to a marked eosinophilic level (1285/cmm.) by the eighth day of refeeding (Table 2).

DISCUSSION

A classical method of inducing lymphatic tissue involution has been that of inanition and has been described extensively (Jolly, 1914; Hayashi, 1924; Jackson, 1925). Jolly (1914), in studying organ weight loss during inanition, demonstrated a loss in weight of lymphatic tissue which far exceeded the loss of body weight. Several investigators (Dustin, 1923; Undritz, 1924; Jackson, 1925; Gaetano, 1928) have reported histological

changes in lymphatic tissue during inanition which consist of diminution in numbers of lymphocytes, decreased numbers of mitoses and the appearance of phagocytic cells. These findings are confirmed quantitatively by the mitotic indices presented here. In addition, a corresponding lymphopenia has been observed during fasting in many species of animals and in man (Tausk, 1896; Keuthe, 1907; Kalmark, 1911; Salkind, 1915; Streicher and Emmel, 1925; Suzuki, 1925; Sjövall, 1936).

As found by previous investigators, the data presented here demonstrate that when intact mice are fasted, the lymphatic tissue loses proportionately more weight than that of the total body. In a comparative study of different lymphatic organs of intact-fasted and intact-refed mice, it was observed that individual organ weights differ with respect to rate and degree of weight loss and weight reconstitution.

Adrenalectomy 48 hours after starting the fasting period resulted in not only an arrest of lymphatic tissue involution but an increase of lymphatic tissue weight. The decreased mitotic index of lymphatic tissue cells observed in intact-fasted mice was particularly marked in the thymus. In spite of the continued fast following adrenalectomy the mitotic rate of lymphatic tissue cells equalled or surpassed that mitotic rate found in the lymphatic tissue cells of intact-fed mice. This reemphasizes the previous findings of Dougherty and White (1945) that adrenocortical hormones diminish lymphatic tissue not only by destroying lymphocytes, but also by inhibiting mitosis. Thus, in spite of the lack of exogenous source of protein, mitosis of lymphocytes occurs in the adrenalectomized-fasted animal.

It is obvious that the amino acids necessary for the synthesis of protein of lymphocytes must be derived from other than lymphatic tissue structural proteins in fasted-adrenalectomized animals. Conversely it has been pointed out previously that adrenocortically mediated acute involution of lymphatic tissue provides a source of protein for other tissues (White and Dougherty, 1947). Regardless of the source (exogenously or endogenously derived protein) the disposition of amino acids necessary for varied degrees of growth of lymphocytes is mediated quantitatively by graded amounts of adrenocortical secretions.

The fact that the thymus is more sensitive to stressors than other lymphatic organs is well established (reviewed by Dougherty, 1952). The data presented here also indicate that the thymus weight did not return to normal levels during the refeeding period as rapidly as other lymphatic organs. This greater susceptibility during acute involution and slower rate of reconstitution of the thymus is likely due to the fact that there is a greater inhibition of mitosis in this lymphatic organ than in others. The slower rate of reconstitution is a consequence of the few reticulo-endothelial

cells in the thymus as compared to the lymph nodes. Thus, there is little opportunity for heteroplastic development of lymphocytes in the thymus during a period of growth following acute involution. An additional factor which may account for differences in rate of involution among the lymphatic organs is that the small lymphocytes which predominate in the thymus are more susceptible to the lymphocytolytic effects of adrenocortical hormones than the large immature reticular lymphocytes which are frequent in the other lymphatic organs (Dougherty and White, 1945). Further, it was recently demonstrated that the larger mature lymphocytes are more resistant to adrenocortical mediated lymphocytolysis than the small mature cells of the blood (Frank and Dougherty, 1953).

The body weights of the previously starved-intact mice returned to normal values much more rapidly than the weights of their lymphatic organs. Conversely, the body weights of previously starved, then adrenalectomized and refed animals did not return to the control values within the experimental period, whereas the lymphatic tissue weight rapidly returned to the level found in control-fed animals of the same age. The differential rates of reconstitution of lymphatic organs of the refed-intact animals was abolished by adrenalectomy.

The nitrogen content per unit of lymphatic tissue weight remained the same in intact-fasted-refed mice and in adrenalectomized-fasted-refed mice. Therefore, it is evident that adrenocortical secretions moderate the total amount of available lymphatic tissue protein but not the amount per individual lymphocyte.

It is suggested that the fasting-refeeding method of study for determining rates of involution and reconstitution of lymphatic tissue can be used as a method for the bio-assay of compounds which produce lymphatic tissue involution and also of compounds and/or nutritional factors which are necessary for lymphatic tissue growth.

SUMMARY

The influences of fasting and refeeding on lymphatic tissue of intact and adrenalectomized CBA mice were studied.

The percentage loss of lymphatic tissue weight of intact-fed mice far exceeded the percentage loss of body weight during a 96-hour fast. Individual lymphatic organs differed in rate and degree of involution. The mitotic rate of lymphatic tissue cells was inhibited by inanition. Adrenalectomy during an uninterrupted fast arrested the involution of lymphatic tissue and resulted in a higher mitotic rate than that found in intact-fed mice.

The body weight of intact-refed mice underwent a more rapid reconstitution than the weight of lymphatic organs, whereas the lymphatic organs

of adrenalectomized-refed mice reconstituted more rapidly than the body weight. Individual differences in rate of lymphatic organ reconstitution were abolished by adrenalectomy.

The nitrogen content per unit weight of lymphatic tissue remained the same in both intact and adrenalectomized mice during fasting and refeeding.

These data support the suggestion that lymphatic tissue protein is made available for metabolic use by adrenocortical mediation. It is also suggested that the fasting-refeeding method of study can be used as a method for the bio-assay of compounds and/or nutritional factors which produce either involution or growth of lymphatic tissue.

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THE INFLUENCE OF THE LIVER UPON ESTROGEN-PROTEIN BINDING *IN VITRO*[1]

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THE liver has long been regarded as the major site of inactivation of the estrogens and other steroid hormones (*cf.*, Pincus and Pearlman, 1943; Pearlman, 1948). Recent evidence has, however, suggested that this organ may perform a dual role in estrogen metabolism, functioning both in the "activation" and inactivation of these compounds (Roberts and Szego, 1947). The mechanism of this activation may involve the elaboration by hepatic tissue of the circulating estrogen-protein complex, *estroprotein* (Roberts and Szego, 1946; Szego and Roberts, 1946).

Investigations of the capacity of surviving liver and other tissues to promote the binding of estrogen to the serum proteins have been made possible by the availability of estrone-16 C^{14} of high specific activity[2] (*cf.*, Heard *et al.*, 1951). The low order of magnitude of the substrate concentration in the present experiments is in sharp contrast to that employed by previous investigators, and may be considered a closer approximation to the physiological levels likely to be encountered *in vivo*. In addition, the choice of serum as the incubation medium represents a departure from previously reported studies in which buffered salt solutions have been utilized. In the course of the present investigation, it was found that rat hepatic tissue was capable of catalyzing the conjugation of estrone or its metabolic products with the proteins of the serum medium. This binding was accentuated in the presence of actively regenerating liver. Surviving spleen, kidney and uterus were completely inactive in this regard.

MATERIALS AND METHODS

The incubation studies were uniformly conducted in rat serum containing estrone-16 C^{14}. The animals supplying the serum and tissues for these studies were female Sprague-Dawley rats, aged 4 to 6 months, which had been maintained on Rockland

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[2] Professor R. D. H. Heard generously placed at the disposal of the investigator a sample of pure crystalline estrone-16 C^{14} prepared in his laboratories. This compound is now being distributed by the Charles E. Frosst Co., Montreal.

Laboratory chow with a supplement of lettuce twice weekly. Serum was obtained immediately prior to use by exsanguination from the dorsal aorta under light nembutal anaesthesia.

In a typical experiment, the isotopically labeled steroid[3] in 95% ethanol stock solution was pipetted into a volumetric flask and the solvent evaporated. Ethanol was then added, in an amount adequate to redissolve the estrogen but not to exceed a final concentration of 1% when serum was subsequently added. After mixing the serum with the isotopic steroid in the flask, 3 ml. aliquots were pipetted into 20 ml. beakers. Each beaker contained 5.82 μg. steroid, equivalent to 10,200 cpm added C^{14}. To some of the vessels were added approximately 150 mg. of tissue obtained from a female rat exsanguinated under nembutal anaesthesia immediately before tissue excision. Regenerating liver was obtained 48 hours after partial hepatectomy. Liver, spleen, and kidney tissue were prepared for incubation by free-hand mincing with a single-edged razor blade. Uterine tissue was not minced. Instead, the two horns were split lengthwise and then cut into several transverse pieces. The cervical portion of the uterus was discarded.

The beakers were incubated at 37.5 to 38° C. for ½, 1, or 2 hours under a constant stream of 95% oxygen—5% carbon dioxide. Most of the studies were carried out in a Dubnoff metabolic incubator, but some of the earlier experiments were performed in Warburg vessels lacking the center well. The results were similar in both instances. At the end of the incubation period, the vessels were placed in a crushed ice bath and the contents of each transferred with medicine droppers to chilled centrifuge tubes. The tubes were centrifuged at 2–4° C. for 5 minutes at 3000 rpm to separate tissue, when present, from the serum medium. The proteins of the decanted serum were then immediately precipitated by dropwise addition to 150 ml. acetone at 0° C. with mechanical stirring. All incubated samples, and non-incubated control samples as well, were take to this point without delay. When liver was present, the tissue remaining in the centrifuge tube was subsequently homogenized and treated as described in Figure 1b. To avoid possible dissociation, due to dilution, of any estrogen-protein complex which may have formed (Roberts and Szego, 1946; Szego and Roberts, 1946) the tissue was not washed before extraction. The first ether extract of the liver, therefore, contained some material rightfully belonging in the serum medium. The techniques employed for the extraction of lipide material present in the serum and tissue proteins (Fig. 1) were adapted from the procedures described by Szego and Roberts (1947) for the determination of protein-bound blood estrogen.

Radioactivity measurements of the residues from the fractions obtained by the above procedures were carried out as follows. All ether extracts were evaporated to dryness, redissolved in 95% ethanol, and plated on aluminum planchettes. Aqueous residues were evaporated and directly plated from water. These procedures appeared to be justified by the fact that aliquots of the same material yielded reproducible radioactivity measurements. In both cases, evaporation of the solvent during plating was aided by use of an infrared lamp separated from the sample by a distance sufficient to avoid charring. Radioactivity was measured in a Tracerlab windowless flow counter. Correction curves for self-absorption in these samples were prepared as follows. Measured amounts of estrone-16 C^{14} in 0.1 ml. ethanol were placed on a series of planchettes. To these were added either varying weights of a preparation of rat liver lipides in benzene, or of purified rat serum proteins in water. The solvents were evaporated in the usual fashion. The self-absorption curve obtained with the liver lipides was used for correcting the observed

[3] The specific activity of this material as determined in the Tracerlab Gas Flow Counter was 1,754,000 counts per min./mg.

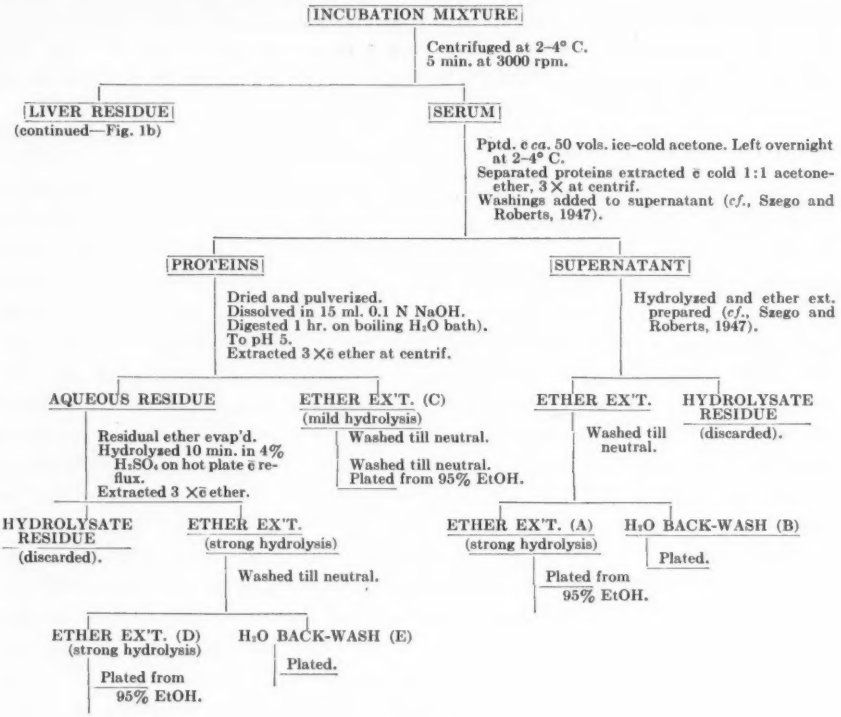

FIG. 1A

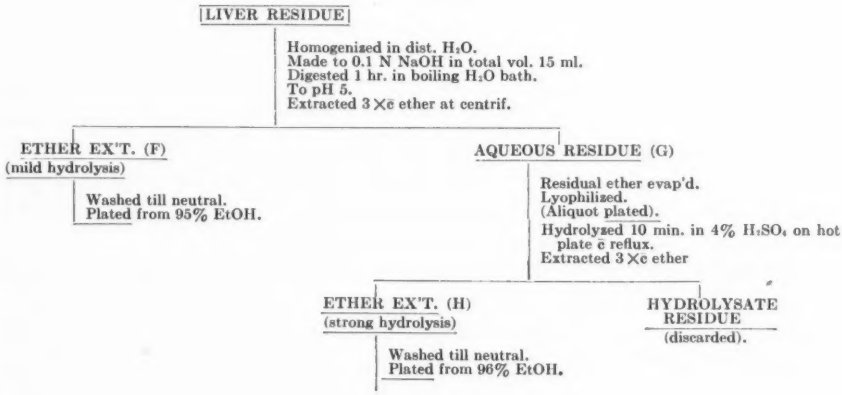

FIG. 1B

FIG. 1a,b. Flow sheet representing fractionation of samples following incubation. Notations for fractions (A) through (H) as in Table 1 and text.

radioactivity in all ether extracts. That obtained with the serum proteins was similarly used for correcting radioactivity measurements on the aqueous residues. The two correction curves were of a similar order of magnitude. The majority of the determinations were carried out on infinitely thick samples (i.e., 4–5 mg./cm.^{2}).

RESULTS AND DISCUSSION

The recovery of C^{14} activity in the various fractions of the incubation medium and of normal and regenerating liver tissue is indicated in Table 1. It will be observed that the fractionation scheme employed permitted the recovery of an average of 88% of the total radioactivity added. This was the case even though respiratory $C^{14}O_2$, which may have been formed during the course of the incubation, was not measured. In addition, neither the serum proteins nor the supernatant portion of the medium was analyzed for possible residual radioactivity following separation of the fractions indicated. Finally, certain fractions normally containing up to 10% of the added activity were not analyzed in some of the experiments (Table 1). In view of these factors, the over-all recovery would appear to have been essentially complete.

It will be noted that neither the presence of liver tissue, nor variation in the time of incubation, had a consistent effect upon the over-all recovery of C^{14} activity. These observations do not rule out some degradation of the original steroid under these conditions, since a significant degree of radioactivity was noted in the aqueous back-wash fractions of a number of ether the extracts, particularly those derived from the serum proteins (Table 1; B, E). The radioactivity present in this water-soluble material appeared directly related to the radioactivity in the ether extract itself (*cf.*, A, D), and in some cases even exceeded the latter. The radioactivity of serum fraction E (possibly representing estrone breakdown products) was negligible in the experiments with spleen, kidney, and uterus but accounted for as much as 10% of the added C^{14} in incubation experiments with regenerating liver. It should be noted, however, that two of the samples of serum incubated 1 hour without tissue also exhibited considerable activity in this fraction, notwithstanding the fact that the activity in the corresponding ether extracts was negligible.

Although surviving liver had no detectable effect on the over-all recovery of added C^{14}, the distribution of isotope among the fractions of the incubation medium was markedly altered in the presence of this tissue. The bulk of the activity was present, apparently unmetabolized, in the serum supernatant ether fraction (A) in all experiments. The presence of both control and regenerating liver consistently diminished the proportion of activity in this fraction, in a degree roughly proportional to the time of incubation. The activity disappearing from this fraction was found in the

TABLE 1. THE INFLUENCE OF SURVIVING LIVER TISSUE ON THE DISTRIBUTION* OF C^{14} ACTIVITY FOLLOWING

TABLE 1. THE INFLUENCE OF SURVIVING LIVER TISSUE ON THE DISTRIBUTION* OF C^{14} ACTIVITY FOLLOWING INCUBATION OF ESTRONE-16 C^{14} IN A SERUM MEDIUM

Hours of incub.	Serum supernatant			Serum proteins				Liver				Grand total
	Ether extract (A)	H_2O backwash (B)	Total	Ether soluble (mild hydrolysis) (C)	Ether soluble (strong hydrolysis) (D)	H_2O backwash (E)	Total	Ether soluble (mild hydrolysis) (F)	Aqueous Residue (G)	Ether soluble (strong hydrolysis) (H)	"Total"[2] (F+G)	
						Serum Control						
0	88.43	1.25	89.68	0.23	0.21	0.32	0.76					90.44
	84.31	1.54	85.85	0.81	0.09	0.05	0.95					86.80
½	121.57	0.0	121.57	0.56	0.05	0.23	0.84					122.41
1	77.35	0.05	77.40	0.15	—	—	>0.15					>77.55
	78.33	—	>78.33	0.26	—	—	>0.26					>78.59
	84.80	—	>84.80	0.21	0.26	3.30	3.77					>88.57
	85.10	—	>85.10	0.32	0.21	2.78	3.31					>88.41
	71.08	2.74	73.82	0.59	0.15	—	>0.74					>74.56
2	97.84	0.61	98.45	0.93	0.15	—	>1.08					>99.53
	87.45	—	>87.45	0.92	0.07	0.06	1.05					88.50
	120.49	0.13	120.62	0.46	0.12	—	>0.58					>121.20
						Normal Liver						
0	75.88	0.77	76.65	0.14	0.05	0.12	0.31	4.57	0.81	0.06	5.38	82.34
	67.55	1.70	69.25	0.61	0.12	0.13	0.86	2.64	2.14	0.25	4.78	74.89
½	63.63	1.00	64.63	0.32	0.24	0.13	0.69	8.80	2.45	0.56	11.25	76.57
1	55.88	0.34	56.22	0.28	— (8.05)[1]	—	>8.33	6.63	6.02	—	>12.65	>77.20
	66.67	—	>66.67	0.36	—	—	>0.36	4.41	5.44	—	>9.85	>76.88
	82.84	—	>82.84	0.32	1.02	—	>1.34	12.00	4.17	0.53	16.17	>100.35
	65.59	—	>65.59	0.24	1.00	3.21	4.45	11.10	5.68	0.57	16.78	>86.82
	83.04	1.03	84.07	0.35	1.02	1.02	2.39	8.93	2.38	1.02	11.31	97.77
2	81.18	0.75	81.93	0.92	2.40	—	>3.32	8.76	3.53	0.52	12.29	>97.54
	46.86	1.24	48.10	0.90	1.70	5.30	7.90	4.56	8.29	0.67	12.85	68.85
	64.20	3.50	67.70	0.60	4.27	5.48	10.35	—	—	—	—	—
						Regenerating Liver						
0	94.02	0.16	94.18	0.25	0.26	—	>0.51	6.86	—	0.21	>6.86	>101.55
	80.10	0.31	80.41	0.14	0.0	—	>0.14	3.61	1.48	0.0	5.09	>85.64
½	89.22	0.65	89.87	0.33	0.65	—	>0.98	8.96	4.35	1.36	13.31	>104.16
	78.33	0.51	78.84	0.33	0.77	—	>1.10	10.60	4.35	0.98	14.95	>94.89
1	55.29	0.37	55.66	0.35	1.59	—	>1.94	8.13	6.53	1.85	14.66	>72.26
	67.65	0.75	68.40	0.34	2.94	—	>3.28	8.30	5.79	1.44	14.09	>85.77
	59.70	1.25	60.95	0.32	2.10	1.78	4.20	—	—	—	—	—
	42.30	4.48	46.78	0.39	2.22	3.72	6.33	—	—	—	—	—
2	66.28	0.74	67.02	0.66	7.50	—	>8.16	7.11	8.24	1.38	15.35	>90.53
	67.06	1.59	68.65	0.57	7.49	—	>8.06	5.04	4.07	1.73	9.11	>85.82
	50.20	0.64	50.84	0.61	4.88	—	>5.49	4.51	5.96	1.11	10.47	>66.80
	59.50	0.39	59.89	0.48	5.67	7.87	14.02	—	—	—	—	—
	42.70	4.14	46.84	0.62	6.01	10.45	17.08	—	—	—	—	—

* For fractions designated, see text and Figure 1. Values indicated are per cent of added C^{14} recovered (of 10,200 cpm≅5.82 μg. added per 3 ml. total medium at 0 time).
[1] Aqueous residue from first ether extraction (mild hydrolysis).
[2] "Total" ether represents, in part, adsorbed material from serum medium (present in ether-soluble fraction after mild hydrolysis). See text.

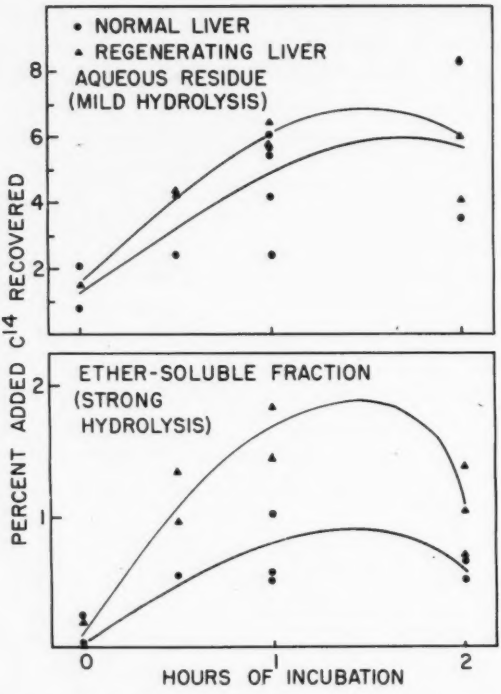

FIG. 2a,b. Recovery of C^{14} in liver tissue fractions following incubation with estrone-16 C^{14} in a serum medium. Figure 2a (upper chart), represents fraction G (cf., Fig. 1; Table 1). Figure 2b (lower chart), represents fraction H. Circles, normal liver; triangles, regenerating liver obtained 48 hours after partial hepatectomy.

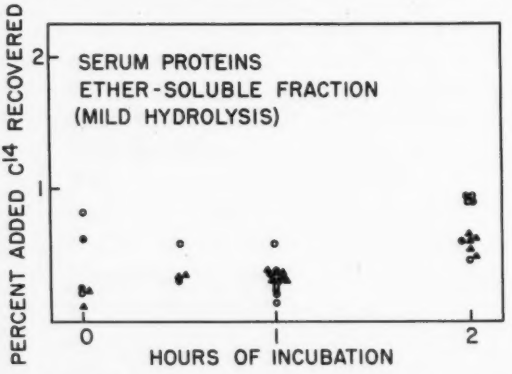

FIG. 3. Recovery of C^{14} in the ether-soluble fraction of the serum proteins following mild alkaline hydrolysis (fraction C). Open circles, serum incubated with estrone-16 C^{14} in the absence of liver; closed circles, normal liver; triangles, regenerating liver.

proteins of the medium and in the liver tissue itself. The association of C^{14} with normal and regenerating liver tissue during incubation is shown in Figure 2. Figure 2a is a schematic representation of the radioactivity remaining associated with liver components (fraction G), following preliminary mild hydrolysis and removal of ether-soluble material (F). It should be pointed out that the C^{14} activity of the latter was thought to represent, in part, material adsorbed on the tissue and perhaps rightfully to be considered derived from the serum medium. Figure 2a reveals that

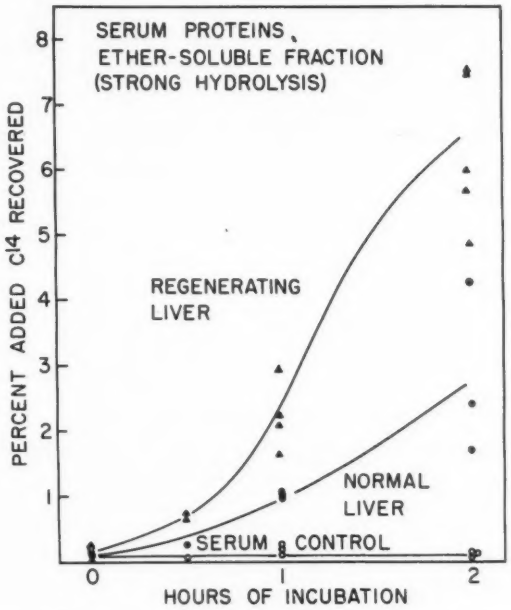

FIG. 4. Recovery of C^{14} in the ether-soluble fraction of the serum proteins following strong acid hydrolysis (fraction D). Notations as in Figure 3.

significant activity was present in the aqueous residue of the liver under these conditions. This activity appeared to increase with time of incubation and was higher in the presence of regenerating liver than normal liver. About one-third of the activity in this fraction could be released as ether-soluble substances after strong acid hydrolysis (Fig. 2b). In the latter instance, unincubated control samples were essentially inactive. An apparent maximum degree of binding was observed after $1\frac{1}{2}$ hours of incubation.

When the proteins of the serum medium were subjected to mild alkaline hydrolysis, relatively small amounts of radioactivity were liberated as ether-soluble material (fraction C). Figure 3 indicates that no significant

differences occurred in the C^{14} activity of this fraction of the proteins of rat serum incubated alone for 0 to 2 hours, or incubated with normal or rapidly regenerating liver for these same periods of time. It would appear that the inconsequential amount of estrogen or its metabolic products present in the ether-soluble fraction after mild hydrolysis of the serum

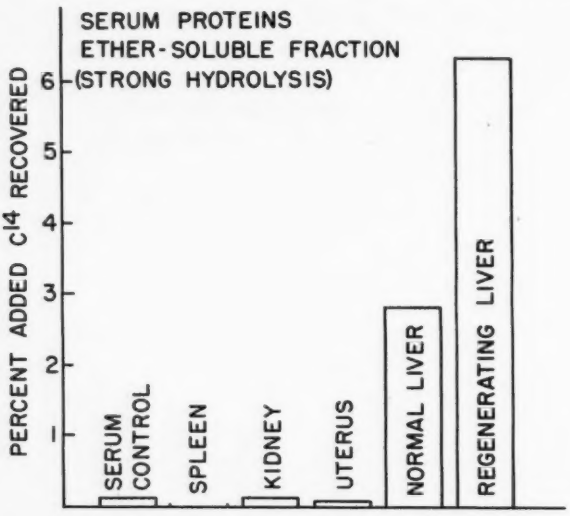

FIG. 5. Incorporation of C^{14} into the serum proteins after incubation of estrone-16 C^{14} with various tissues. The ether-soluble fraction (D) following strong acid hydrolysis is shown, as in Figure 4. All incubations were carried out for 2 hours. The height of the bars for serum control, normal and regenerating liver samples represents an average of 3, 3 and 5 samples, respectively. The values for the other tissues are the result of single determinations.

proteins was not an index of the estrogen-metabolizing activities of the liver.

In contrast, secondary strong acid hydrolysis of the aqueous residue remaining after ether extraction of the mild alkaline digest liberated significant quantities of lipide-soluble C^{14} activity (fraction D). The radioactivity in this fraction varied significantly with the nature of the tissue and the length of the incubation period (Fig. 4, 5). This radioactive material presumably represented estrone or its metabolic products in firm combination with the serum proteins. Insignificant amounts of firmly bound radioactivity were present in the proteins of serum which had been incubated alone for 0 to 2 hours. In the presence of normal hepatic tissue, this firmly bound material increased markedly during the 2-hour incuba-

tion period. Regenerating liver, which is known to be hyperactive in many of its metabolic characteristics (*cf.*, Szego and Roberts, 1949), was extremely active in binding estrone or its metabolic products to the serum proteins *in vitro*. The capacity of surviving liver to bind radioactive material derived from estrone-16 C^{14} to the serum proteins appeared to be highly specific for this tissue. It will be observed in Figure 5 that spleen, kidney, and uterus of the rat were completely inactive in this respect.

These observations suggest that protein-binding of estrogen or its metabolic products is a physiological process, presumably catalyzed by a specific enzyme system found in liver but not in spleen, uterus, or kidney. The nature of the substances which become firmly attached to the serum proteins during incubation with surviving hepatic tissue is not definitely known. A lipide material is indicated by the solubility properties. The fact that a relatively large fraction of the original estrogen added was recovered in this form suggests that the structure may still be steroidal. That at least a portion of this material was still estrogenic was indicated by preliminary results on bioassay of this fraction. Only when liver tissue had been present in the original incubation did fraction D exhibit appreciable estrogenic activity. Other chemical properties indicate that this material was probably bound to the serum proteins in an esterified (or glucuronide) form, and, in fact, the major role of the liver in facilitating estrogen-protein binding may be by virtue of its ability to catalyze glucuronide formation (*cf.*, Crépy, 1947). On the basis of available evidence, *estroprotein*, the natural circulating estrogen, appears to be constituted of estrogen linked to serum protein in this esterified (or glucuronide) form (Roberts and Szego, 1946). The results of the present study are consistent with the hypothesis that the liver may be the site of formation of this *estroprotein*.

SUMMARY

Estrone-16 C^{14} was incubated in the presence of surviving rat tissues in homologous serum for periods varying from 0 to 2 hours. The distribution of radioactivity was studied in various fractions of the medium and tissues. Approximately 88% of the activity added could be recovered in these fractions. Normal liver tissue was capable of enhancing the binding of estrone or its metabolic products to the proteins of the serum medium. The protein-bound C^{14} activity was released in an ether-soluble form only after partial hydrolysis of the proteins. Regenerating liver was even more active in promoting binding of the steroid to the serum proteins. Spleen, kidney, and uterus were completely inactive in this regard. The relation of these observations to the problem of *estroprotein* formation *in vivo* is discussed.

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CHEMICAL TRANSFORMATION OF STEROIDS BY ADRENAL PERFUSION: PERFUSION METHODS[1]

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INTRODUCTION

STUDIES were undertaken in 1944 to discover conditions requisite for the maintenance of corticosteroid biosynthesis in isolated, perfused adrenal glands. In 1949, having developed a perfusion apparatus (Hechter, 1948) and methods for extracting and estimating corticosteroids in blood, it was possible to demonstrate that perfused beef or sheep adrenals respond to adrenocorticotrophic hormone (ACTH) by a marked increase in the rate of corticosteroidogenesis (Hechter, 1949). Possessing an active adrenal preparation, studies were then undertaken to evaluate the enzymatic capacity of perfused adrenals to modify steroids. In the course of these studies using 11-desoxycorticosterone (DOC), suggestive evidence was obtained that the gland possessed a system for directly introducing an oxygen function at C-11 of the steroid nucleus.

A few months later the dramatic therapeutic effects of cortisone in rheumatoid arthritis were reported by Hench, Kendall and their associates (1949). Since our findings represented a possible mode for the introduction of a C-11 oxygen function into the steroid nucleus, the work on adrenal perfusion was considerably expanded. This rapidly led to the definitive chemical characterization of corticosterone (Hechter *et al.*, 1949) as the DOC transformation product responsible for the glycogenic activity previously noted in adrenal perfusates. Further studies were then under-

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taken to isolate and characterize the transformation products resulting from the perfusion of a variety of C-19 and C-21 steroids through adrenals. Some of these findings have been discussed in a review in which it was not possible to give detailed descriptions (Hechter *et al.*, 1951).

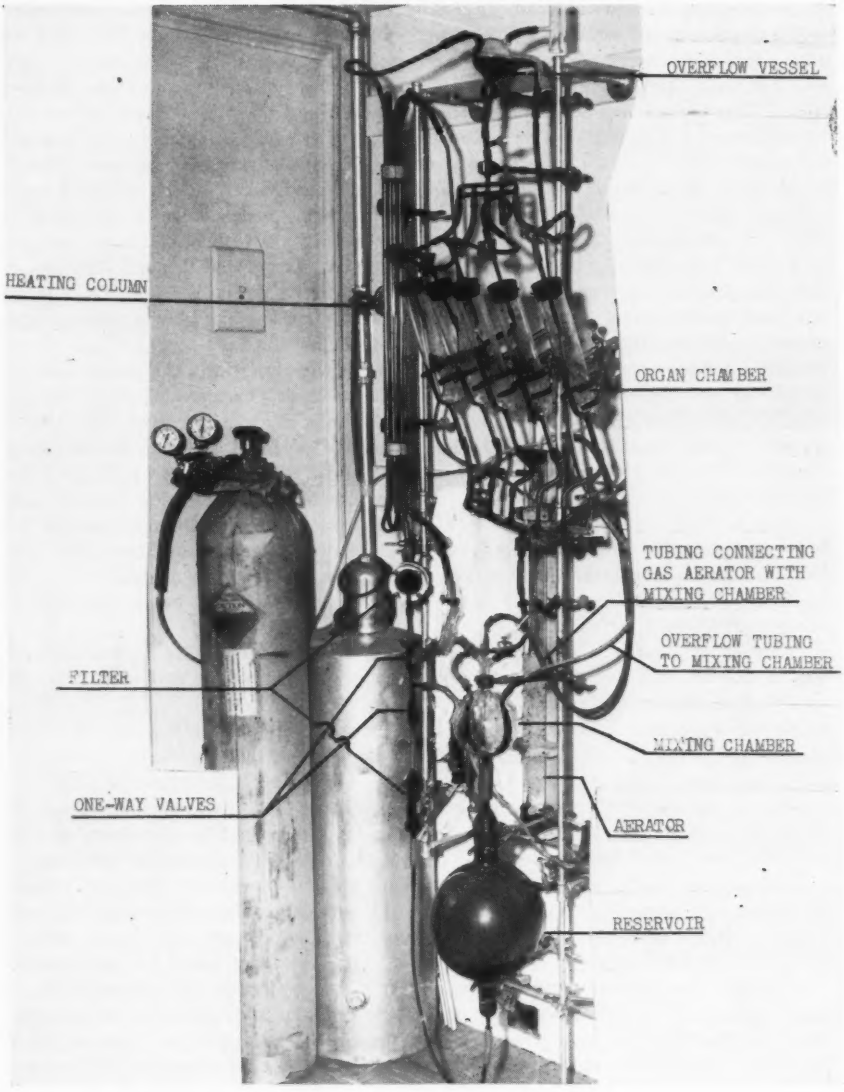
This paper is the first of a series on steroid transformations induced by isolated, perfused bovine adrenal glands and describes in detail methods of adrenal perfusion. The development of the adrenal perfusion technic to its present point was carried out primarily by studying the conversion of DOC to corticosterone; thus the data to be presented will deal mainly with the 11-bio-oxygenation system using DOC as a substrate.

METHODS

Technic of arterial perfusion of bovine glands: In the initial phase of this study, the methods developed originally for the demonstration of ACTH-induced corticosteroidogenesis (Hechter, 1949) were employed. Bovine glands were obtained at a local slaughter house. The animals available were mainly cows which for various reasons had gone off milk or heifers which had failed to breed. In a few cases, bull adrenals were obtained. It should be emphasized that the adrenals employed were derived from animals of variable age and weight, some with minor bovine diseases, whose previous nutritional regime may be safely assumed to be quite variable.

The glands were obtained after the animals had been stunned, bled out, skinned and eviscerated, approximately 15 to 30 minutes after exsanguination. With the carcass hanging on the dressing rack, one adrenal, usually the left (which is kidney-shaped in contrast to the crescent-shaped right adrenal) was removed within 1–2 minutes with all the adjacent periadrenal connective tissue, together with the abdominal aorta and vena cava. Initially, attempts were made to perfuse the gland through one of the several adrenal arteries. However, the adrenal vascular supply in bovines was found to be so highly variable that this technic was not consistently successful. Thereafter, for arterial perfusion, glands were perfused through the aorta as follows: A large cannula was inserted through one end of the abdominal aorta; a glass plug was tied into the other end distal to all adrenal vessels and all of the large extra-adrenal arteries were ligated. Particular care was taken not to disturb the fat and connective tissue surrounding the gland. The vena cava was slit open and the principal adrenal vein of the gland (in some cases there are 1 or 2 additional small veins) was cannulated, the wall of the vena cava being used as a flap to tie the venous cannula in place. The remainder of the vena cava was then carefully dissected away and the preparation was thoroughly perfused with saline or Tyrode's solution (20–40 min. after obtaining the gland) to remove all residual blood and to detect major leaks, which were then tied off. This preparation of the adrenal gland was carried out in the slaughterhouse. The glands were then taken to the laboratory, about 25 minutes away. Initially, during this interval before the glands were placed in the apparatus, they were perfused with chilled (5° C.) plasma by gravity at a pressure of 70 mm. Hg. Later it was discovered that equally good results were obtained when the preparation was transported without perfusion, but merely covered with cold Ringer's solution.

Preparation of blood for perfusion: Beef blood was collected non-aseptically at a local slaughterhouse. Large-mouthed gallon bottles containing 600 ml. of glucose-citrate solution (2.06% sodium citrate, 0.78% citric acid, and 5.0% glucose) were used for blood



Perfusion apparatus

collection from the severed neck vessels of stunned cattle. After collection, the blood was brought to the laboratory and 100,000 units of penicillin G and 500 mg. of streptomycin sulfate per liter were added to inhibit bacterial growth and the blood was stored at 2–5° C. for 1 to 7 days prior to being used for perfusion.

On the day of the experiment, the refrigerated blood was filtered first through 6–7 layers of muslin, then through a nylon stocking filter (Fenwal). This treatment removed

gross clots, agglutinated red cells as well as certain scums of an amorphous character which develop in bovine blood collected and stored under our conditions. The blood was then circulated through a heterologous liver used as a biological filter designed to remove small thrombi and foreign material which might have passed through the previous filters. This preliminary perfusion treatment, in addition, removes vasoconstrictor substances present in stored blood or plasma. Instead of using homologous lung as in previous work (Hechter, 1948), rat liver was employed for this purpose as follows: The filtered blood was added to the perfusion apparatus, mounted in a 38° C. incubator room, and permitted to circulate. While the blood was being warmed and oxygenated, an etherized rat weighing 200–300 g. was exsanguinated by severing the femoral arteries and veins. The chest was then opened and a cannula was inserted through the right auricle into the thoracic vena cava directed toward the liver. The residual blood in the liver was then rapidly washed out by perfusion with physiological saline at pressures not exceeding 40 mm. Hg. The abdominal vena cava below the liver was next tied off and the liver was then removed from the animal. With this procedure. the portal vein and hepatic artery are left open and the circulation proceeds "backwards" from the vena cava→hepatic vein→portal vein (mainly) and hepatic artery. The liver was then inserted into the organ chamber of the apparatus and perfused with the medium to be used for subsequent perfusion through cattle glands. Rat livers were perfused either with pulsatile or non-pulsatile pressure (in either case the mean perfusion pressures were 30–45 mm. Hg.), depending upon whether the adrenal to be perfused subsequently was to be perfused with pulsatile or non-pulsatile pressure. Under our conditions, the blood flow through the livers was 40–60 cc. per minute and the blood was circulated for varying intervals, but at least twice through the liver preparatory to being employed for subsequent adrenal perfusion.

Addition of steroids to perfusion media: DOC and other steroids were completely dissolved in minimal volumes of propylene glycol and then added dropwise to rapidly swirled, liver-filtered perfusion blood. DOC is quite soluble in propylene glycol and was employed at a concentration of 50 mg. per ml. in this vehicle. With less soluble steroids such as 17-hydroxy DOC, Δ^5-pregnen-3β-ol-20-one, etc., it was necessary to use correspondingly more propylene glycol. Using such propylene glycol solutions of DOC, it is possible to prepare DOC suspensions in blood as high as 1 gm./liter before large particles of DOC separate (after 24–48 hours). However, this should not be interpreted to indicate that DOC concentrations lower than 1 gm./L. of blood represent true solutions.

Table 1 shows an experiment in which a beef gland was arterially perfused through the aorta with plasma containing successively increasing concentrations of DOC with a mean pulsatile perfusion pressure of 125 mm. Hg. It will be seen that upon perfusing the gland with DOC at a concentration of 30 mg./L., the adrenal blood flow is unchanged from that of the control period. When the DOC concentration was increased to 100 mg./L., there was a tendency for the blood flow to decline, and this became marked when the DOC concentration was increased to 300 mg./L. After the flow had been slowed by the latter concentration, no increase in blood flow aws observed when the DOC concentration was lowered from 300 to 30 mg. DOC/L., demonstrating that the lowering of blood flow produced by high concentrations of DOC was irreversible. It should be emphasized that none of these plasma samples containing DOC concentrations ranging from 30 to 300 mg./L. showed any turbidity. Presumably the colloidal suspension produced by 300 mg./L. DOC of plasma was so broken (possibly in the capillaries during the fluid exchanges between plasma and tissues) that mechanical blockade of about two-thirds of the vascular channels by insoluble steroid resulted.

TABLE 1. INFLUENCE OF DOC CONCENTRATION UPON ADRENAL BLOOD FLOW*

Time of perfusion, min.	Blood flow, ml./min.	Pressure, mm. Hg	Remarks
5	31	125	At 0 min., perfusion initiated with plasma, no DOC
10	36	125	
20	32	125	
40	36	125	
45	33	125	After 40′, perfusion initiated with 30 mg. DOC per L.
50	32	125	
60	31	125	
80	32	125	
85	31	125	After 80′, perfusion initiated with 100 mg. DOC per L.
90	28	125	
100	26	125	
120	27	125	
125	24	125	After 120′, perfusion initiated with 300 mg. DOC per L.
130	16	126	
140	14	125	
160	10	125	
165	8	125	After 160′, back to 30 mg. DOC per L.
170	6	125	
180	5	125	
200	7	125	At 200′, perfusion pressure increased to 200 mm. Hg.
205	19	200	
210	22	200	
200	26	200	
230	25	200	
240	23	200	

* Gland weight = 18.2 gm.

As with DOC, other steroids added to blood likewise block adrenal vascular channels at particular concentrations. In the case of each steroid, the concentration at which this occurs must be determined empirically, for it has not been possible to predict the manner in which a steroid will perfuse through an adrenal. Perhaps this depends on the nature of the metabolic products formed during adrenal perfusion, although other possibilities such as the nature and size of the steroid colloid micelles formed may be involved. For example, Δ^5-pregnen-3β-ol-20-one can be successfully perfused at 25 mg./L. whereas with concentrations of 50–100 mg./L., flow is partially blocked. Progesterone, while more soluble in propylene glycol than pregnenolone, may be perfused under our conditions without significant blockade in the range of 50–70 mg./L., but produces block at 100 mg./L. Nevertheless, Δ^4-androsten-3,17-dione, which is not significantly more soluble than progesterone in propylene glycol, has been perfused through glands at a concentration of 100 mg./L. without difficulty.

Arterial perfusion of DOC: The degree of DOC conversion into corticosterone by arterially perfused glands is shown in Table 2. In these experiments (and in those described in succeeding tables) the analytical figures for corticosteroids isolated from perfusates refer to fractions obtained by silica gel chromatography. The isolation procedure and the method of fractionation by chromatography will be described fully in a subsequent paper. Column (a), Table 2, refers to recovered crystalline starting material melting above 130°; column (b), to the crystalline product, corticosterone, melting above

160°; and column (c) to the weight in DOC equivalents of formaldehydogenic steroid (FS) present in the mother liquors of the crystallized fractions.

It will be seen from Table 2 that the rate of formation (expressed as mg. of corticosterone/hr./20 gm. gland) and the percentage yield of crystalline corticosterone vary from 3.4 mg. to 30.5 mg. and 5% to 23%, respectively, in seven experiments. The 11-oxidation of DOC occurs with plasma as well as with mixtures of blood and plasma.

The arterial perfusion method has several serious limitations which are not evident from the data of Table 2. These are: (a) The preparation of adrenals for arterial perfusion, demanding a high degree of surgical skill, could not be standardized because of variations from gland to gland. (b) A high percentage of the preparations developed serious leaks in the aorta during the course of experimentation, necessitating termination of the experiment. (c) Perfusion of glands with a variety of dyes after the experiment had been completed revealed that some areas of the gland were inadequately circulated and,

TABLE 2. 11-HYDROXYLATION BY BOVINE GLANDS PERFUSED *via* AORTA

Expt. No.	Perfusion conditions						Perfusate analysis			Rate†
							Crystalline			
	Gland wt., gm.	DOC input, mg./L.	Medium	Mean arterial pressure, mm. Hg.	Mean blood flow, ml./min.	Perfusion period, min.	DOC (a) %	Cort. (b) %	Non-cryst. F.S., %	
1	18.5	30	Plasma	125	27	64	38	8	23	4.3
2	20	50	Plasma	120	7	222	24	16	20	3.4
3	24	50	Bl. Mixture*	100	8	167	22	23	17	4.6
4	12	70	Plasma	100	21	48	23	10	35	14.7
5	12	70	Bl. Mixture*	110	27	112	39	8	15	15.1
6	13	100	Plasma	190	22	340	32	15	11	30.5
7	19	100	Plasma	130	23	44	41	5	16	7.3

* Refers to equal volumes of plasma and whole blood.
† mg. of corticosterone per hour of perfusion per 20 gm. gland.

in some adrenals, these ineffectively perfused areas represented one-third to one-half of the gland. (d) It was difficult to control blood flow at will, thus some preparations exhibited subnormal blood flow at high perfusion pressures, while in others, despite low perfusion pressures, the flow was relatively rapid. (e) Finally, arterially perfused preparations contained a great deal of extra-adrenal tissue (which tended to complicate any results obtained) which could not be dissected away without injury to the adrenal arterial vessels running through them.

Accordingly an attempt was made to develop a simpler method adaptable to routine standardization whereby these difficulties could be reduced or improved.

Venous perfusion: Consideration of the circulation of the adrenal suggested that venous perfusion might remedy some of the above problems. Since there is a single large adrenal vein which empties directly into the vena cava, this vessel could easily be cannulated within 1–2 minutes. By perfusing *via* the vein, fluid was delivered first into the venous lumen, then through the medulla, next the cortex, and finally out the innumerable adrenal arteries at the surface of the gland, a reversal of the normal process. In this type of preparation, all of the periadrenal fat and connective tissue were superfluous and could be rapidly dissected away. The entire preparation required no more than 5 minutes and little surgical skill. Venous perfusion of glands had previously proved to be successful in a preliminary study (Hechter, 1949) and accordingly a systematic investigation of venous perfusion was undertaken.

It was found that when glands were cannulated for venous entry of the perfusion medium the blood flow was exceedingly slow despite utilization of mean pulsatile perfusion pressures as high as 200 mm. Hg. In Table 3 are shown the data of 6 experiments using glands perfused via the adrenal vein. It will be seen that such preparations 11-

oxygenate DOC when blood was present in the perfusion medium (Expts. 4, 5 & 6), but in marked contrast to the previous results (*cf.* Table 1) no detectable corticosterone was formed when DOC was perfused in plasma as the medium (Expts. 1, 2, & 3).

This difference between media in venous preparations became explicable as the result of visual inspection of glands perfused with whole blood. Following each pulsatile stroke (which pumped fully oxygenated blood into the central venous lumen distending the gland) a pool of dark deoxygenated blood passed back upon recoil from the gland lumen into the glass cannula. Soon a considerable portion of the cannula was filled with deoxygenated blood which surged back and forth as the pulsatile perfusion continued. These observations suggested that the blood flow obtained by venous perfusion of glands was so slow that the oxygen dissolved in the medium was depleted by the adrenal medullary tissues. Thus it appeared likely that with plasma, whose oxygen capacity is limited, the perfusion medium entering the cortical layers was so completely deoxygenated that the bio-oxygenation of DOC could not occur. With blood, however, whose oxygen-carrying capacity is high, sufficient oxygen was retained in the medium to permit the oxidative process to function.

TABLE 3. 11-HYDROXYLATION BY BOVINE GLANDS PERFUSED *via* ADRENAL VEIN

Expt. No.	DOC input, mg./L.	Gland wt., gm.	Medium	Mean pressure, mm. Hg.	Mean blood flow, ml./min.	Perfusion period, min.	Crystalline		Non-cryst. F.S.,	Rate†
							DOC %	Cort. %		
1	40	18	Plasma	150	6.8	338	33	0	25	0
2	50	22	Plasma	100	10.0	200	42	0	12	0
3	100	20	Plasma	100	6.1	155	33	0	12	0
4	50	20	Bl. mixture*	150	7.1	326	4	35	31	7.5
5	70	13	Bl. mixture*	120	11.6	222	6	36	18	27.0
6	100	20	Bl. mixture*	100	10.7	89	30	10	20	6.4

* Refers to equal volumes of plasma and whole blood.
† Refers to mg. of corticosterone per hour of perfusion per 20 gm. gland.

From hemodynamic consideration, it appeared most likely that the slow blood flow with venous-perfused glands resulted from a constriction of the arterioles and small arteries in the cortical layers near and at the surface of the gland. Attempts to increase the flow by means of a variety of vasodilators and by sympatholytic agents were, however, without significant influence.

Venous perfusion: Lacerated glands—pulsatile pressure.—A method of overcoming this difficulty of flow in venous-cannulated preparations presented itself as the result of an accident. In two experiments when the mean pulsatile pressure was increased to about 200 mm. Hg, the gland burst after a relatively short period and blood containing DOC was pumped rather rapidly through the leaks which developed. Considering that the major portion of DOC escaped through the leak without passing through adrenal tissue, it was surprising to find rather high concentrations of corticosterone upon workup of perfusate samples from these burst glands. This observation led us to lacerate with a razor blade the outside surface of the gland to a depth of 2–3 mm. on the basis that such lacerations might provide a by-pass for the escape of blood which had circulated through the adrenal tissue. Table 4 illustrates the relationship between the number of lacerations placed on the gland surface and the adrenal blood flow under a constant pressure head of 90 cm. of H_2O. It will be seen that the flow through the gland (at constant perfusion pressure) initially rises rapidly and then tends to level off as the number of lacerations is increased progressively. Using the venous-lacerated type of preparation, it was soon learned that the steroid conversion rates were higher than those achieved by our previous procedures.

Venous perfusion: Lacerated glands—non-pulsatile pressure.—During the course of

TABLE 4. THE INFLUENCE OF THE NUMBER OF LACERATIONS UPON FLOW THROUGH A VENOUS CANNULATED GLAND*

Lacerations		Flow, ml./min./gm.
Number	Total length, cm.	
0	—	0.11
1	6.1	0.43
2	11.5	1.02
3	16.0	1.43
4	24.0	1.72
5	30.0	2.10
6	35.2	2.42

* A gland weighing 18.7 gm. was perfused with Tyrode's solution at a constant pressure of 90 cm. H_2O. Length of individual lacerations were measured with flexible mm. rule.

perfusing lacerated glands via the vein with pulsatile pressures, it was noted that many glands burst during the course of several hours of perfusion and it was not possible to evaluate blood flow rates. It seemed likely that these tears in the gland resulted from the alternate flexing and relaxing (induced by pulsatile perfusion pressures) of glands whose connective tissue capsule had been effectively destroyed by the lacerations. Accordingly, the pulsatile pressure of the perfusion apparatus was eliminated and the pump was used merely for elevating the blood for non-pulsatile perfusion under suitable hydrostatic pressure. With this technic, hundreds of lacerated glands have been perfused through the adrenal vein and in only an occasional instance has a gland developed "leaks" during perfusion periods for as long as 12 hours. Accordingly, the non-pulsatile perfusion technic became our method of choice. Table 5 illustrates the rate of 11-hydroxylation of DOC in venous-lacerated glands (perfused with pulsatile and non-pulsatile pressures). The high rates of conversion obtained should be compared with results shown in Tables 2 and 3.

TABLE 5. COMPARISON OF PULSATILE AND NON-PULSATILE PERFUSION OF LACERATED GLANDS CANNULATED *via* THE ADRENAL VEIN WITH 100 MG. DOC PER L

Expt. No.	Type	Perfusion medium		Gland wt., gm.	Mean pressure perfusion mm. Hg	Mean bl. flow, ml./min./gm.	Rate*
		% blood	% plasma				
1	Non-pulsatile	10	90	14	28	1.03	32
2	Non-pulsatile	50	50	17	30	1.20	48
3	Non-pulsatile	50	50	15	26	1.15	57
4	Non-pulsatile	100	0	22	60	1.35	48
5	Non-pulsatile	100	0	18	100	1.35	36
6	Pulsatile	10	90	22	19	1.10	48
7	Pulsatile	20	80	20	20	1.00	52
8	Pulsatile	50	50	17	32	1.32	42

* mg. of corticosterone per hour of perfusion per 20 gm. gland.

Laboratory scale production of cortisone by perfused adrenal glands: Since corticosterone, the product isolated from DOC perfusions, was at that time (spring, 1950) unavailable in other than milligram amounts from

adrenal extracts, it was felt important to produce gram quantities of this corticosteroid for biological experimentation. As our objective, production of 5 to 6 gm. of corticosterone was set. A bank of individual glands was perfused with DOC in blood under conditions designed to insure a good yield of corticosterone, no attention being given to the rate of production. From our foregoing experimental work, it was decided to recirculate 200 mg. of DOC/L. of blood through a bank of 5 glands, each with its own entry tube from a manifold. By proper adjustment of stopcocks, it was possible to regulate adrenal blood flow to equalize large differences in flow rate between individual glands so that all glands would have a blood flow of about 1.5–2.0 L. per hour, independent of gland weight.

Table 6 shows the results of five consecutive experiments using 20 to 28 L. of blood. In experiment A, the perfusate was circulated once through two 5-gland banks, in the remaining runs (B to E), the perfusate was circulated 2 to 3 times through 1 such bank. The average yield of crystalline corticosterone produced under these conditions was 37%, based on the weight of the DOC recovered from the perfusate. In total, 5.63 gm. (34%) of purified corticosterone was obtained from the 16.6 gm. of desoxycorticosterone disappearing from 108 L. of perfusate.

TABLE 6. THE PRODUCTION OF CORTICOSTERONE BY BANKS OF ADRENAL GLANDS PERFUSED WITH DOC

Run No.	Perfusate vol., liters	Input DOC, gm.	Cryst. unaltered DOC, gm.	Cryst. cort., gm.	% cryst. cort.*
A	28	5.5	0.83	1.84	39
B	20	4.0	1.21	0.92	33
C	20	4.0	1.12	1.05	36
D	20	4.0	0.83	1.23	39
E	20	4.0	0.88	1.16	37

* Based on the weight of desoxycorticosterone recovered.

In considering these results, it should be emphasized that approximately half of the added steroid is unaccountable as either crystalline DOC or corticosterone by our isolation method.

DISCUSSION

An example of the perfusion method guided exclusively by biochemical principles has been given. Conditions of perfusion have been selected solely on the basis of the rates of an *in vitro* reaction, and not upon physiological considerations. Approached in this fashion, a simple technique for perfusion of bovine adrenals (as well as for the adrenal glands of sheep, pig, horse and human, the other species tested) has been developed which requires probably less skill than is necessary for the preparation of adrenal

slices. Since our findings may be generally applicable to a variety of problems in organ metabolism, some aspects of these studies merit discussion.

Organ transfer from the animal to the perfusion pump: The present work demonstrates that in the case of the specific enzymatic activity studied, the C-11 hydroxylation system, it is not necessary to transfer the organ rapidly from the animal to the perfusion pump. Glands are routinely perfused which have been obtained from animals killed several hours prior to experimentation. Indeed, glands stored at 2°C. in salt solution and perfused 24 hours after the death of the animal are still capable of transforming DOC to corticosterone, albeit at a somewhat reduced rate. It should be emphasized that the stability of the C-11 "oxidase system" described is not a special case of an "exceptional" enzyme. The entire series of biochemical events involved in the formation of corticosteroid hormones and cholesterol from C-2 fragments, involving a multiplicity of enzymes coupled with the appropriate energy-yielding reactions, also proceeds in such glands perfused hours after the death of an animal (Hechter, *et al.*, 1951) (Zaffaroni *et al.*, 1951).

It should be emphasized that all glands were stored in salt solutions at or near 0°C. The temporary anoxia involved in gland storage of concern to some physiologists who believe that organs should be transferred to the artificial circulation as rapidly as possible to minimize anoxia, ignores the point that the dimensions of anoxia involve temperature as well as time. The question as to whether an organ stored near 0°C. is anoxic cannot be answered *a priori* but requires a determination of the oxygen requirements for the maintenance of the system at that temperature. It seems likely that a great variety of enzymatic processes in tissues other than the adrenal will prove to be sufficiently stable (at low temperature) to allow time for an unhurried surgical preparation of the organ for perfusion. Where a highly unstable constituent is a component of the reaction under investigation, it may be possible to overcome such deficiencies by preliminary perfusion with blood, with presumable resynthesis of the "unstable" component.

Preparation of organs for perfusion: Ordinarily, one thinks of perfusing an organ through the artery since this is the normal course of circulation. Biochemically, however, it is of no fundamental concern whether substrates are presented to cells from the venous or the arterial side of the capillary. The present study provides an illustration of this principle in the case of an organ wherein "normal" arterial perfusion is very difficult from a surgical point of view, but where the venous cannulation is simple. The difficulties in blood flow exhibited in this preparation were overcome by lacerating the cortex of the gland. While this treatment undoubtedly damages cells, quantitatively the total number of cells so injured is vanishingly small relative to such injury in tissue slices. Application of these considerations to the preparations of other organs will undoubtedly remove many of the surgical difficulties formerly associated with perfusion. Finally, it

should be mentioned that organs of small laboratory animals with blood vessels too small for easy cannulation may be replaced by slaughterhouse material, allowing the use of numerous organs from a variety of species.

Perfusion media: We have previously shown that a variety of perfusion media may be employed to demonstrate 11-hydroxylation of DOC in bovine adrenals and that, of these, homologous beef blood seems to be the most satisfactory (Hechter *et al.*, 1951). This should not be taken to indicate, however, that physiological media are necessary to obtain maximal activity of a particular metabolic system. The blood used in our studies, while homologous, contained abnormally high concentrations of glucose, citrate and antibiotics. Citrated blood was originally used in our studies when it became prohibitively expensive to employ the "more physiological" anticoagulant, heparin. As it developed subsequently, citrated blood may well be superior to heparinized blood for the study of a number of adrenal corticosteroidogenic processes. The use of homologous blood in our present experiments represents an accident of convenience since both beef glands and blood were available. In other studies it has been shown that bovine blood is as effective as sheep blood in ACTH-induced steroidogenesis in sheep glands (Hechter, 1949). The use of heterologous blood in organ perfusion represents a solution of those problems which arise when the organs perfused are derived from animals which cannot easily supply adequate volumes of homologous blood. As an illustration of this point, it may be mentioned that in steroid metabolic studies of perfused rat livers carried out by one of us (O.H.), bovine blood has proven especially useful as a convenient perfusion medium on a 20 L. scale.

Role of perfusion in biochemical investigation: We have previously stated that a perfused organ may be likened to an organized collection of tissue slices wherein the circulatory apparatus is employed to permit entry of substrates into the cells and have discussed some of the factors which influence the rate of biochemical reactions in a perfused organ (Hechter, *et al.*, 1951), *e.g.* capillary hydrostatic pressure, permeability of substrate through the capillary wall, possibility of change in the number of effective vascular channels, etc. These added complexities of the perfusion technic, difficult to control quantitatively, limit the usefulness of the perfusion method.

What, then, is the place of perfusion in modern biochemical investigation? In our opinion, the present study affords one clearly useful example. Although corticosteroidogenesis has long been regarded as involving a series of transformations presumably starting from cholesterol, the metabolic pathways involved and the end products were subjects of speculation until our perfusion work provided a basis for the beginning of an understanding of the intermediaries and enzymatic systems involved (Hechter, *et al.*, 1951). The present report is concerned with one step in this process, the 11β-hydroxylation of DOC, and illustrates perfusion as a method for obtaining sufficient material to enable the definitive chemical characterization of an unknown metabolic product derived from a specific substrate. Once products of metabolic reactions are known, moreover, it becomes possible in many cases to device suitable micromethods for the study of

active systems by simpler *in vitro* methods. Thus, following our demonstration of the 11-hydroxylation of DOC, confirmation of this reaction in adrenal slices, breis, and homogenates was soon accomplished (Hayano *et al.*, 1949) (Savard *et al.*, 1950) (McGinty *et al.*, 1950) (Haines, 1952). It seems clear that a detailed characterization of the enzymatic system involved will not significantly be facilitated by perfusion work but requires enzymological techniques. In similar fashion, other reactions described in the perfused adrenal have likewise been demonstrated in homogenates such as the 17- and 21-hydroxylation of steroids (Hayano and Dorfman, 1952) (Plager and Samuels, 1952), the conversation of Δ^5-3β-hydroxy to Δ^4-3-ketosteroid (Samuels *et al.*, 1951), and the condensation of C-2 fragments from acetate to corticosteroid hormones (Haines *et al.*, 1951) (Haynes *et al.*, 1952). The conversion of cholesterol to corticosteroid hormones has not as yet been achieved in simpler systems; studies involving the latter system in perfused glands will continue to be useful until this system is achieved in homogenates or slices. While the examples cited above are drawn from a single field, corticosteroid biosynthesis, we suggest that the application of the perfusion method to the study of the metabolism of a variety of biologically active substances is bound to be profitable.

Having discussed areas wherein perfusion may prove important, one general aspect of perfusion relative to other *in vitro* methods should be mentioned. With both slices and homogenates following the addition of substrate, the products of reaction accumulate. These products may either inhibit the rate of reaction or, depending upon specific conditions, may be further converted into secondary and tertiary products in a "normal" or "abnormal" pathway. Moreover, as the reaction proceeds, the level of substrate declines continuously from its original concentration. Perfusion without recirculation of the perfusate provides an *in vitro* metabolic system wherein substrates can be administered at a constant concentration during the course of experimentation and products of reaction may be removed continuously in a manner comparable to the *in vivo* situation.

SUMMARY

A description is given of the development of methods of perfusing isolated beef adrenal glands using 11-desoxycorticosterone as the substrate for the gland's 11-oxygenating system. A study of the perfusion of DOC in various media by arterial and venous perfusion has led to the use of gravity flow through lacerated beef glands *via* the adrenal vein. The limitations of the perfusion method for the study of the enzymatic systems involved in corticosteroidogenesis as well as its use as a tool in general biochemical investigation are discussed.

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β-GLUCURONIDASE STUDIES IN INBRED MICE: ANDROGENIC HORMONES AND KIDNEY AND URINARY β-GLUCURONIDASE ACTIVITY[1,2]

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INTRODUCTION

THE effects of sex hormones on tissue β-glucuronidase activity in inbred mice were reported in a previous paper (Fishman & Farmelant, 1953). Among other findings, it was established that testosterone propionate produced a marked elevation in kidney β-glucuronidase in all inbred strains which were studied. In some preliminary histochemical experiments, it appeared that the enzyme was localized in both the lumina and in the epithelial cells lining the renal tubules. This suggested that some of the androgen-stimulated kidney β-glucuronidase may be excreted in the urine. The present paper deals with experiments designed to study this possibility.

METHODS AND EXPERIMENTAL PROCEDURES

Details may be found in a previous publication (Fishman and Farmelant, 1953) concerning types and source of animals used, the nature of the hormone treatment, types of controls, method of assay of β-glucuronidase activity, etc.

The routine of urine collection was as follows. Often at two times in the day, 10 A.M. and 4 P.M. and always once each day, 4 P.M., the mice were induced to urinate into Wassermann test-tubes, by gentle pressure with the fingers on the bladder area. A 1 to 10 dilution was prepared (0.1 ml. urine+0.9 ml. H_2O) ordinarily, but 1 to 50 and 1 to 100-fold dilutions were required for the assay of β-glucuronidase-rich urines. When the amounts of urine were insufficient, specimens of two or more mice in the same group were pooled.

A period of incubation was selected in which not more than 5% of the substrate phenolphthalein glucuronic acid present in the digest was hydrolyzed (1 hour for β-glucuronidase-rich and 18 hours for β-glucuronidase-poor urines). The amount in micrograms of phenolphthalein liberated was measured (Fishman, Springer and Brunetti, 1948) and the urinary β-glucuronidase (phenolphthalein units) expressed in terms of 1 hour incu-

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bation of undiluted urine (100 ml.). No difference in urinary β-glucuronidase was found in urines, one aliquot of which was assayed immediately and another aliquot being allowed to remain stoppered in the refrigerator for as long as 3 days before assay. The pos-

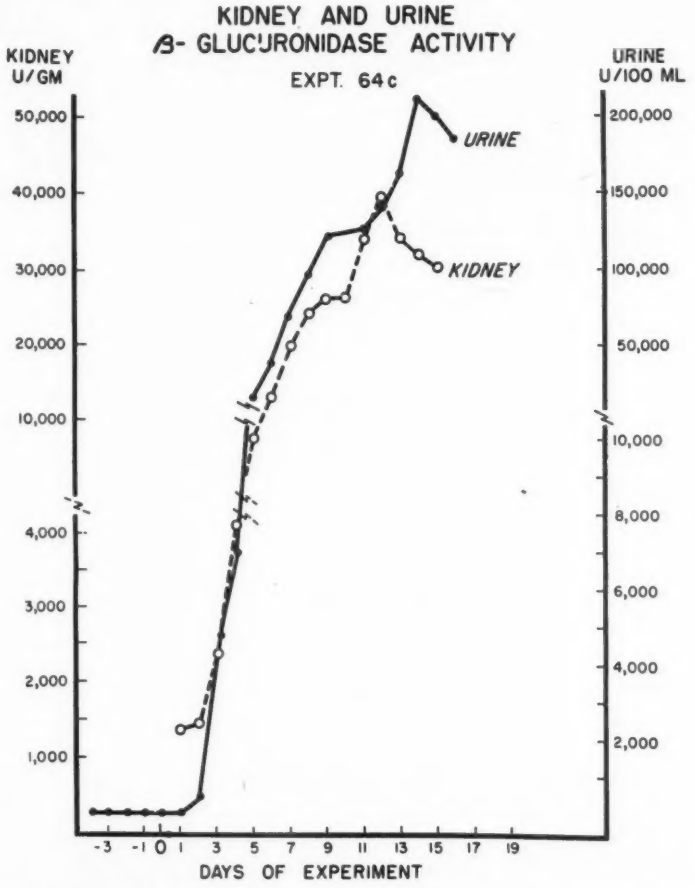

FIG. 1. Kidney and urinary β-glucuronidase activities in DBA female mice injected for 16 days with 2.5 mg. daily of testosterone (Oreton F, *Schering*, aqueous suspension). Each white circle represents toe average of β-glucuronidase values of the kidneys of 3 mice and each black circle, the β-glucuronidase activity the value of fresh pooled urine of the surviving animals at the corresponding experimental day.

sibility of contamination of the urine by β-glucuronidase-rich bacteria is considered remote on the basis of negative control experiments with toluene-preserved and non-preserved urines. The design of the experiments is readily understood from the material presented under results.

TABLE 1

PART I. URINARY β-GLUCURONIDASE ACTIVITY OF FEMALE DBA MICE

Days of R_1*	Individual Mouse Values (Units/100 ml.) Animal Nos.												
	1	2	3	4	5	6	7	8	9	10	11	12	Pooled Urine Data†
−7	0	0	0	0	0	0	0	0	0	0	0	0	
−6	0	0	0	0	0	0	0	0	0	0	0	0	
−3	0	0	0	0	0	0	—	—	—	—	—	—	
−2	—	0	—	—	—	—	—	0	—	0	0	0	
−1	—	0	—	0	0	0	0	0	—	0	0	—	
+3	965	603	664	664		0	0	664	664	0	0	956	
+4	2,710	2,760		4,030	1,900	2,250	3,510	4,020	3,570	4,500	2,130	4,500	
+5		5,180		9,840		5,120	9,840	12,200		15,600		9,400	
+6	21,700	10,600	11,400	22,800	18,950		24,400		27,300		18,350		
+7	20,200	11,390	9,690	27,300	25,400		21,600	25,420	22,680		20,700		26,900 (6, 10)
+8										45,800	35,500	46,500	42,600 (3, 9)
													47,100 (1, 2, 7, 8)
+9			90,000			86,000	83,000			138,000			154,000 (4, 11, 12)
+10	102,000			170,000		81,000	140,000	172,000	122,000	140,000	150,000	195,000	129,000 (2, 3, 5)
+11	187,000	105,000	123,000										180,000 (4, 5, 6, 11)
+12	150,000	100,000	130,000	134,000		100,000	134,000				144,000		214,000 (5, 9, 10, 12)
+13		92,000	140,000	220,000	140,000	120,000	282,000					209,000	203,000 (1, 9, 10)
+14		90,000					130,000						185,000 (9, 10)
													138,000 (1, 5,8)
													125,000 (6, 11)
+17		118,000	129,000		166,000	122,000							217,000 (1, 4, 9)
													209,000 (7, 8, 12)
+19	206,000	152,000	136,000	180,000		185,000		236,000	195,000	187,000	168,000	185,000	
+21			172,000			254,000							292,000 (10, 11)
													270,000 (1, 2, 4, 5)
+24	207,000	132,000	172,000						146,000		113,000		233,000 (1, 10, 12)
													147,000 (6, 8)
+25				312,000						220,000	136,000	190,000	163,000 (5, 6)
													236,000 (3, 9)

PART II. TISSUE β-GLUCURONIDASE ACTIVITY (UNITS/GM.) OF FEMALE DBA MICE

Days of R_1*	Mouse	Kidney	Liver	Spleen
26	9	32,400	6,030	4,350
26	12	45,400	7,300	4,560
27	10	36,700	6,520	5,580
27	11	38,000	7,050	6,140
28	5	44,200	4,450	4,650
28	8	43,200	5,800	3,590
56	2	50,300	6,390	5,100
56	4	42,500	—	—
60	1	58,500	6,900	5,810
60	6	57,400	6,530	4,940
	Controls			
	1	1,260	4,300	5,630
	2	1,770	3,480	5,400
	3	1,670	6,370	6,860

* Each of 12 DBA female mice 3 months old received 2.5 mgm. of an aqueous suspension of testosterone propionate daily for 60 days. Urine was collected once daily when possible for 25 days after injections began.

† Insufficient specimens were pooled for β-glucuronidase assays. The numbers in parenthesis identify the mice supplying this urine.

RESULTS

Urinary and renal β-glucuronidase in androgen treated mice. Results of a representative experiment of this study are given in Table 1. Urinary β-glucuronidase concentration was determined in individual female DBA mice from 7 days prior to and during 25 days of daily injections of testosterone propionate. It may be noted that in the control period the urines were negative (43 specimens from 12 mice collected individually over a 6-day period). Positive indications of the excretion of β-glucuronidase were found in eleven mice by the fourth day of hormone administration. In the case of each of the 12 mice, the urinary β-glucuronidase levels continued to rise as a rule. These rose rapidly in the first days and less so in the later period of observation. Six of these mice, sacrificed at 26, 27 and 28 days exhibited renal β-glucuronidase activity much greater than normal. By 60 days of treatment, the renal β-glucuronidase was still high.

Time relationships in the androgen-induced renal and urinary β-glucuronidase response. Essentially the same experimental conditions were followed as in the experiment described in the preceding paragraph, except that groups of several mice were sacrificed at regular intervals in order to measure the kidney β-glucuronidase activity during the 16-day period of testosterone administration (Fig. 1). Both kidney and urine β-glucuronidase exhibited significant increases on the third day of the experiment and the rate of increase in enzyme activity for both was similar.

Histochemical localization of renal β-glucuronidase in androgen-stimulated mice. The method of Friedenwald and Becker (1948) utilizing the substrate, 8-OH, quinoline glucuronic acid, was applied to thin sections of kidneys taken from mice treated with androgen. Plate I illustrates the localization of the stain, representing β-glucuronidase activity, in the tubules of the cortex. This section is representative of many such from experiments on androgen-stimulated kidneys.

DISCUSSION

In attempting to evaluate the experimental evidence concerning the possible excretion in the urine of androgen-stimulated renal β-glucuronidase, it is of interest to review information concerning kidney and urinary β-glucuronidase. Two phenomena have been described; one is the sex difference of kidney β-glucuronidase noted in certain strains of inbred mice (Morrow, Carroll and Greenspan, 1951, Fishman and Farmelant, 1953) and the other is the low renal glucuronidase of C3H mice (Morrow, Greenspan and Carroll, 1949, Fishman and Farmelant, 1953). It is found in this laboratory: (1) that in strains showing sex differences (e.g. DBA, White Swiss) urines from male mice were almost always richer in β-glucuronidase than those collected from female animals and (2) that C3H mice

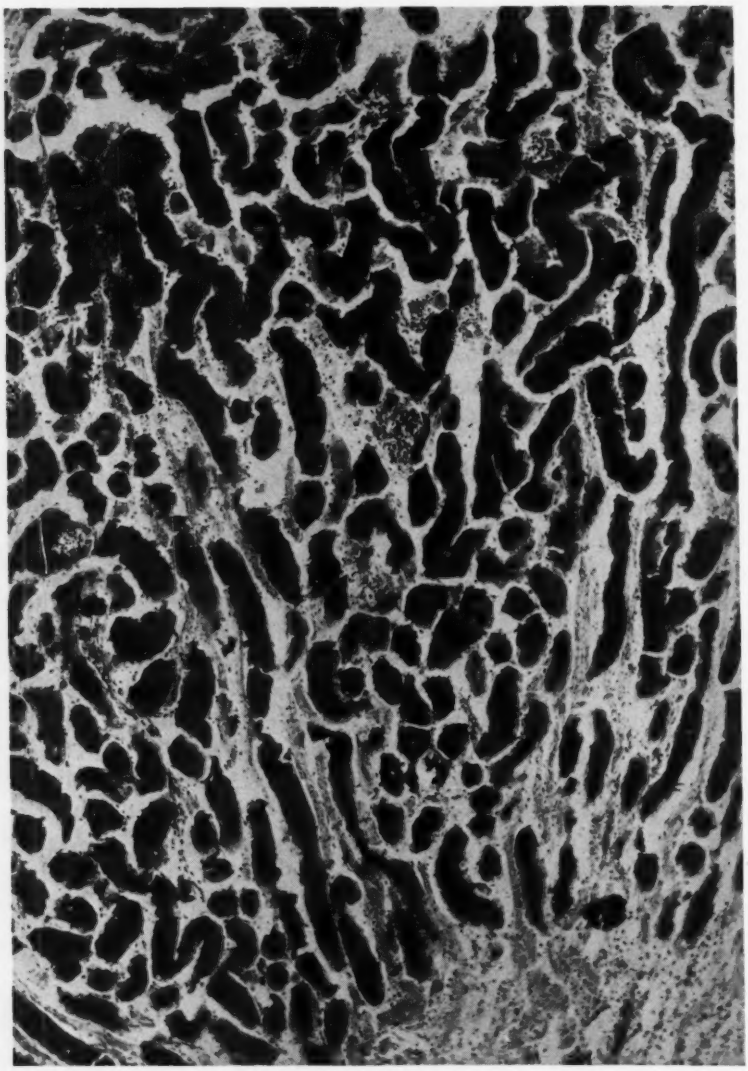

PLATE I. Localization of β-glucuronidase in the kidney of a C3H male mouse treated with testosterone-propionate for 30 days. Period of incubation with 8-OH quinoline glucuronide 18 hours. β-Glucuronidase activity 14,000 units/gram. Magnification 400×. Section is 10μ in thickness.

with very few exceptions excrete no urinary β-glucuronidase in contrast to the high β-glucuronidase mice (DBA and White Swiss mice, particularly of the male sex) (Riotton and Fishman, unpublished).

The present data demonstrate that the administration of testosterone

propionate to mice induces an increase in β-glucuronidase of the renal tubules with an augmented level of this enzyme appearing in the urine. In addition to the studies in female DBA mice, qualitatively identical results were observed in male White Swiss mice given testosterone propionate or methylandrostenediol and in C_3H male mice receiving testosterone propionate.

An insight into the mechanism of the urinary excretion of β-glucuronidase in animals given androgen is gained from Figure 1 which shows the time relations in β-glucuronidase activity for kidney and urine. Thus, it is clear that β-glucuronidase in significant amounts is found in the urine not before but on the first day an increase in kidney β-glucuronidase was observed. It appears that the enzyme finds its way from the kidney tubules directly into the urine without delay and suggests a phenomenon localized to the genito-urinary apparatus.

Many histological preparations of kidneys from androgen-treated animals did not reveal pathological changes. Although the urine of DBA and other mice normally contains protein in small amount, it was possible to detect the excretion of abnormal amounts of protein in the urine of androgen-treated animals. The question of how much of this protein is β-glucuronidase remains to be answered.

A discussion of the relevance of other important considerations, such as the renotrophic effects of androgens, to the phenomena reported in this and the previous paper is being reserved for a later communication.

SUMMARY

Testosterone propionate increased by substantial amounts the excretion of β-glucuronidase in the urine of mice. This phenomenon was accompanied by the simultaneous elevation of β-glucuronidase activity in the kidney tubules. Under the conditions of the experiments, significant augmentation in both renal and urinary β-glucuronidase of androgen-treated DBA mice was observed by the third experimental day.

Acknowledgments

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THE INACTIVATION OF INSULIN BY LIVER SLICES OF THE RAT[1]

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THE demonstration that extracts of rat liver contain an insulin-inactivating system (insulinase) (Mirsky and Broh-Kahn, 1949) and an inhibitor of this system (insulinase-inhibitor (Mirsky, Simkin and Broh-Kahn, 1950) raises the possibility that the rate of insulin inactivation within the intact cell is determined by the balance of activity resulting from the mobilization of either of the two systems. Since the assay of the enzymatic activity of extracts may not represent the "working" activity of the cell prior to disintegration by homogenization, it was deemed essential to investigate the inactivation of insulin by relatively intact cells such as comprise surviving slices of liver.

METHODS

Fed male rats of from 150 to 200 gm. in weight were decapitated and as much blood as possible was drained from the carcass of each animal. The liver was removed and placed in cold phosphate buffer, pH 7.4. Slices of approximately 1 cm^2 in size were prepared with a Stadie-Riggs apparatus (Stadie and Riggs, 1944). After blotting gently with filter paper, the slices were weighed and the desired amount placed into a Warburg flask containing 2 ml. of the phosphate buffer in the main compartment and 3 units of insulin in 1 ml. of buffer in the side-arm. When insulinase-inhibitor was employed, it was dissolved in the buffer containing the insulin. After gassing for 5 minutes with 95% oxygen+5% CO_2 mixture, the contents of the side-arm were dumped into the main compartment, the flasks were shaken at a rate of 116 cycles per minute and maintained at 37° C. At the end of the incubation period, 1.5 ml. of the incubation mixture, representing an original content of 1.5 units of insulin, were removed for immediate assay. The slices were dried overnight to constant weight.

The inactivation of insulin during the incubation with liver slices was estimated by the effect of the 1.5 ml. aliquots of the incubation mixture on the blood sugar of rabbits fasted overnight. Blood samples were drawn prior to and at 30 and 60 minutes after the intravenous injection of the incubation mixture. The change in the blood sugar concentration was expressed as the percentage of the average of two fasting sugar levels determined prior to injection. The sum of the percentages of the 30 and 60 minutes blood samples were designated as the "pooled percentage of initial blood sugar" which, in turn, served as a semiquantitative estimate of the insulinase activity of the liver slices. Thus, if no insulin were destroyed during incubation, the "pooled percentage" would be low

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whereas if all the insulin were destroyed, the blood sugar level would remain unchanged and the "pooled percentage" would equal 200. Two sets of an equal amount of slices were prepared from each liver and then assayed as above. The mean of the "pooled percentage" of the two rabbits was used as the estimate of the insulinase activity of each liver.

Two sets of controls were performed; one set consisting of slices incubated in buffer without insulin and the other of buffer with insulin incubated as described but without slices.

Amorphous insulin assaying about 22 units per mg.[2] was utilized. The insulinase-inhibitor was prepared from fresh beef livers by the procedure described in a previous communication (Mirsky, Simkin and Broh-Kahn, 1950). The blood sugar concentration was determined by Nelson's method (Nelson, 1944).

RESULTS

The injection of 1.5 ml. of the phosphate buffer after incubation with liver slices in the absence of insulin produced insignificant changes in the blood sugar of rabbits. Ten sets of slices, ranging from 7.3 to 33.7 mg. dry

TABLE 1. INACTIVATION OF INSULIN BY DIFFERENT PREPARATIONS OF EQUIVALENT AMOUNTS OF LIVER

Homogenates prepared with phosphate buffer, pH 7.4. Supernatant prepared by centrifugation of homogenate at 18,000 for 20 minutes. Slices prepared as described in text. Incubation mixture made up to contain equivalent of 250 mg. liver (wet weight) in total volume of 3 ml. phosphate buffer containing insulin. Rabbits injected with volume of mixture which originally contained 1.5 units.

Preparation	Insulin incubated (units)	Incubation period (minutes)	Insulinase activity ("pooled percentage")
Homogenate	12	30	215
Supernatant	12	30	105
Slices	12	120	87
Slices	3	30	108
Slices	3	120	149
Slices	3	180	200

weight, were incubated for two hours and then assayed as described. The resultant mean "pooled percentage" was 204.3 (S.E. $\pm$0.45) which does not differ significantly from that resulting from the injection of 1.5 ml. of untreated buffer. Accordingly, the incubation of liver slices in phosphate buffer does not release any factor which can produce a change in the blood sugar of rabbits.

Assays of 1.5 ml. phosphate buffer containing 1.5 ml. units of insulin after incubation for two hours without liver slices were performed concomitantly with assays of buffer incubated with slices. The mean "pooled percentage of the initial blood sugar" produced by the insulin was 73 (S.E. $\pm$5.7).

In order to evaluate the relative efficiency of homogenates and slices in

[2] We are indebted to the Eli Lilly Company for generous supplies of insulin.

the inactivation of insulin, preparations of equivalent amounts of liver were studied. As is indicated in Table 1, homogenates and the supernatants after centrifugation at 18,000 g. have a greater insulinase activity than do slices of liver.

The relation between the quantity of liver and the inactivation of insulin was determined by incubating insulin with from 1 to 6 slices for 120 minutes. The data obtained in 54 experiments are depicted in Figure 1 from which it is apparent that the greater the quantity of liver, the greater the insulinase activity. In spite of the scatter of the data, the regression coefficient of the insulinase activity ("pooled percentage") on the dry weight of the slices was highly significant ($b = 2.2078$; $P < 0.001$).

The relation between insulinase activity and length of incubation was determined by keeping the quantity of slices constant and varying the period of incubation. The data thus obtained are illustrated in Figure 2 for two different amounts of slices. It is apparent that the rate of insulinase activity, as indicated by the regression of the "pooled percentage" on the time of incubation, is essentially the same for slices weighing 28.0 (S.E. ± 0.9) mg. ($b = 0.5406$) as for slices weighing 61.5 (S.E. ± 1.3) mg. ($b = 0.5853$), although the amount of insulin destroyed at any particular interval is greater with the larger quantity of liver. Analysis of variance revealed that the differences between the activity at 60 and 120 minutes and between 120 minutes and the other intervals were highly significant ($P < 0.001$).

Since fasting produces a marked diminution in the insulinase activity of liver extracts (Broh-Kahn and Mirsky, 1949), it became pertinent to determine the influence of fasting on the activity of liver slices. Figure 3 illustrates the insulinase activity (mean $\pm$ S.E.) obtained with slices from the livers of 40 rats fasted for 72 hours. When slices of less than 30 mg. dry weight were employed no significant difference could be demonstrated between those from livers of fed and fasted rats. With larger quantities of liver slices, however, the activity was significantly higher in those from fasted animals. It is noteworthy that even with the smallest amount of liver, no diminution of activity occurred in slices of liver from fasted rats.

In order to determine the influence of insulinase-inhibitor (Mirsky, Simkin and Broh-Kahn, 1950) on the insulinase activity of slices from the livers of fed rats, 36 experiments were performed with slices incubated for 120 minutes in phosphate buffer containing insulin and slices in buffer containing both insulin and insulinase-inhibitor. The quantity of inhibitor employed in each instance was equivalent to 20 gm. of fresh liver. Figure 4 illustrates the mean $\pm$ S.E. activity for slices grouped according to dry weight. Analysis of variance revealed that within each group of slices the presence of inhibitor produced a highly significant diminution in insulinase

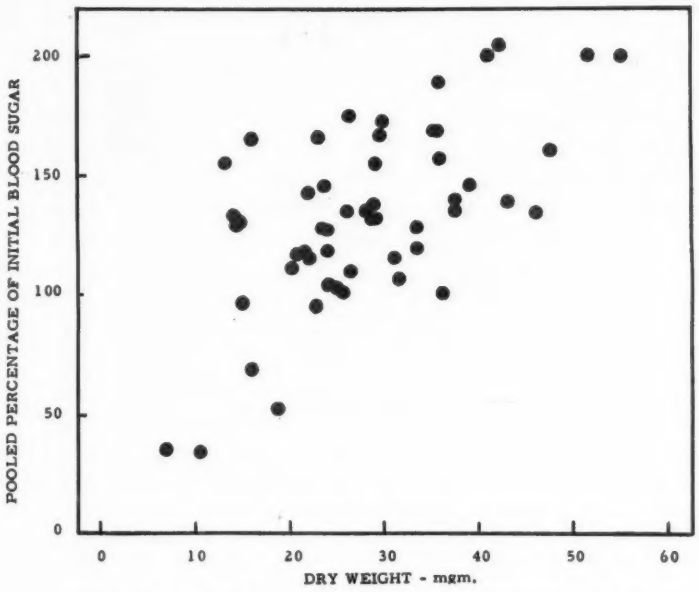

FIG. 1. Relation between the quantity of liver slices and the inactivation of insulin.

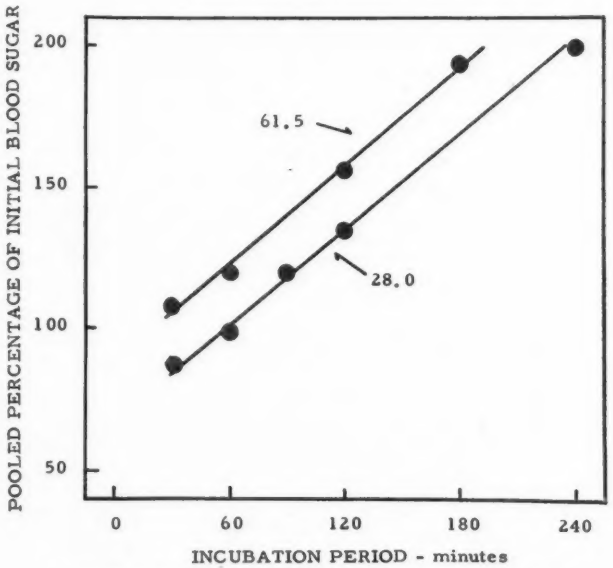

FIG. 2. Relation between duration of incubation and the inactivation of insulin by liver slices.

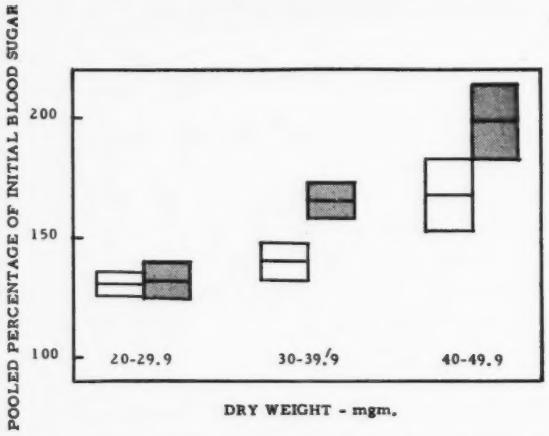

FIG. 3. Effect of fasting on inactivation of insulin by liver slices. The "pooled percentage" (mean ± S.E.) after incubation of insulin with slices from fed rats (open boxes) and with slices from rats fasted 72 hours (cross-hatched boxes).

activity ($P < 0.001$). The same statistically significant difference was established when the dry weights of the liver slices were not grouped and analysis of covariance was employed.

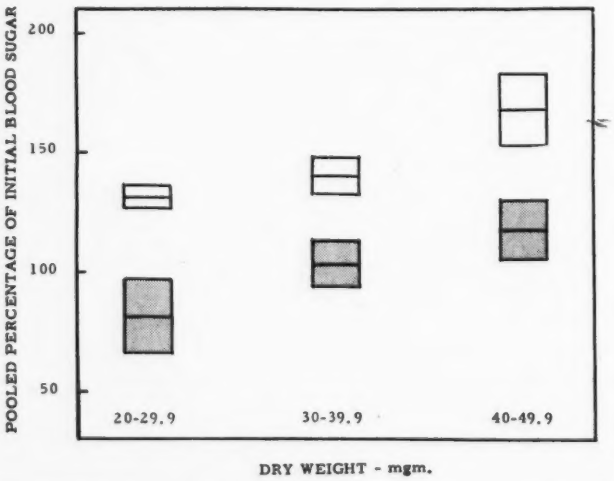

FIG. 4. Effect of "insulinase-inhibitor" on the inactivation of insulin by liver slices. The "pooled percentage" (mean ± S.E.) after incubation of insulin with slices without addition of inhibitor (open boxes) and with slices in presence of inhibitor (cross-hatched boxes).

DISCUSSION

Like homogenates and extracts of liver (Mirsky and Broh-Kahn, 1949), surviving liver slices inactivate insulin *in vitro*. This inactivation is dependent both upon the quantity of liver employed and the length of the incubation period. As with homogenates, the addition of insulinase-inhibitor (Mirsky, Simkin and Broh-Kahn, 1950) to the incubation mixture results in a significant reduction in the insulinase activity of liver slices. With slices, however, the spontaneous rate of insulin inactivation is much slower than with homogenates of equivalent amounts of liver. Consequently, it may be concluded that within the intact cell, only a portion of the potential insulinase is active; the remainder being inhibited by an insulinase-inhibitor.

The insulinase activity of homogenates or extracts from fasted rats is markedly diminished (Broh-Kahn and Mirsky, 1949). Slices of liver from similarly fasted rats, however, exhibit a definite tendency to destroy more insulin than do those from fed rats. Since the inactivation of insulin probably is dependent upon the balance between insulinase and insulinase-inhibitor, the discrepancy between the activity of homogenates and that of slices of livers from fasted rats may be attributed to a greater decrease in the inhibitor than in the insulinase.

It is apparent from the preceding that the assay of homogenates or extracts of liver cannot yield an adequate measure of the rate of insulin inactivation by the intact cell. The slice, however, does provide a measure of the balance between insulinase and insulinase-inhibitor activities, and therefore may be representative of events as they occur *in vivo*. Accordingly, an increase in the rate of insulin inactivation should occur in the intact fasted rat. Since insulin production by the pancreas is decreased during fasting (Best, Haist and Ridout, 1939), the increased rate of insulin inactivation should result in a relative insulin insufficiency. Further, with an increase in insulinase activity, exogenous insulin should be less effective since only a portion of the quantity administered will escape inactivation.

The aforementioned postulates are in accord with established data concerning the phenomenon of "starvation diabetes." Thus, the fasted animal not only responds to the ingestion of carbohydrate with a typical "diabetic tolerance" curve, but also shows a decreased hypoglycemic response to the injection of insulin (Chambers, 1938).

The relevance of insulinase and insulinase-inhibitor to the etiology of diabetes mellitus in man is difficult to evaluate at the present time. The presence of these factors in extracts of liver and the demonstration that they are effective in the intact cell lends support to the hypothesis that

an insufficiency of insulin may result from an increased destruction of insulin either in consequence of an increase in insulinase activity or of a decrease in the availability of the insulinase-inhibitor (Mirsky, 1951).

SUMMARY

The insulin-inactivating activity (insulinase) of slices of liver is much less than that of homogenates or extracts of equivalent amounts of liver.

Insulinase activity is related directly to the quantity of liver slices employed and to the length of incubation.

In the presence of an inhibitor extracted from liver (insulinase-inhibitor), the insulinase activity of liver slices is markedly reduced.

Whereas fasting results in a diminution in the insulinase activity of homogenates and extracts of liver, slices of liver from fasted rats tend to have an increased activity. It is proposed that the insulin-inactivating capacity of liver slices is dependent upon the balance of insulinase and insulinase-inhibitor activities, and that with fasting there is a greater reduction in the inhibitor than in the insulinase.

The phenomenon of "starvation diabetes" may be atrributed in part at least to a relative increase in insulinase activity.

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THE THYROID STIMULATING HORMONE OF THE ANTERIOR PITUITARY AS DISTINCT FROM THE EXOPHTHALMOS PRODUCING SUBSTANCE[1]

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FOR almost two decades it has been known that anterior pituitary extracts when injected into animals produce hyperplasia of the thyroid and exophthalmos. Probably owing to the frequent coexistence of these two features in patients with Graves' disease, it has been generally assumed that the hyperplasia of the thyroid and the exophthalmos in the experimental animals were caused by the same pituitary factor, the thyroid stimulating hormone (TSH).

From observations (Dobyns, 1946a, 1946b) on the exophthalmos producing activity of a variety of TSH preparations, it has become apparent that more striking exophthalmos was produced by some extracts than others although hyperplasia of the thyroid was produced by all. It has been reported by Jefferies (1949) that following the iodination of pituitary extract the thyroid stimulating material was inactivated but some exophthalmos producing activity remained. On the other hand, Dobyns and Rawson (1951) have found that an extract rich in TSH when exposed to thyroid tissue slices *in vitro* lost the capacity to produce both responses.

Albert (1945) has shown that the Fundulus, the common Atlantic "minnow," (*Fundulus heteroclitus, Linn.*) will develop exophthalmos following the administration of small amounts of anterior pituitary extract.

Albert (1945) tested a variety of prepared endocrine substances as well as six anterior pituitary hormones (in various degrees of purification) which he had prepared from pituitaries of sheep. He observed that the exophthalmos producing activity paralleled only the thyrotropic activity and that preparations of follicle stimulating hormone and luteinizing hormone which produced exophthalmos also caused hyperplasia and hypertrophy of the thyroid.

The purpose of this study has been to determine the exophthalmos producing quality of a large number of anterior pituitary fractions which

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had been prepared in a variety of ways and for a variety of purposes and for which some assay data concerning some anterior lobe hormones were available. It became apparent from the study of some preparations that the exophthalmos producing and the thyroid stimulating activities did not necessarily parallel one another. These observations have led to the separation of thyroid stimulating hormone (TSH) from the exophthalmos producing substance (EPS).

METHODS

Groups of 8 to 12 Fundulus were placed in stainless steel tanks. Three to six groups of fish were used in each experiment. One group of fish received normal saline; one group of fish received a standard pituitary extract for which a known exophthalmos producing response had been determined; and the remaining groups received various unknown preparations.

The material to be tested was weighed and then dissolved in normal saline. A single dose of 0.25 mg., or a fraction thereof, in 0.25 ml. of saline was injected intraperitoneally by introducing a #26 needle through the vent and the wall of the bowel. Many fractions and subfractions of pituitary extracts rich in various trophic substances as well as the by-products of purification procedures were tested.

Colored thread was placed through and tied in the lower lip of each fish so that individual identification was possible. The intercorneal distance on each of the fish was measured to 0.1 mm. by means of an improved caliper method modified after that described by Dobyns (1945). Such measurements were made on each fish before and at intervals following the administration of the material tested. The degree of exophthalmos was calculated as the percentage increase in the intercorneal distance in each fish. The average percentage change among the fish of each group was thus obtained.[2]

The amount of TSH in the various preparations was determined in day-old, male, white leghorn chicks by one or several modified methods: 1) the increase in thyroid weight (Bergman and Turner, 1939); 2) the depletion of iodine from the thyroid (Piotrowski *et al.*, 1953); 3) the increase in mean cell height of the thyroid (Rawson and Salter, 1940).

RESULTS

As a result of screening a large number of pituitary fractions, it was observed that a few fractions which contained appreciable amounts of TSH produced relatively little exophthalmos; conversely, a few preparations which contained relatively little TSH produced obvious exophthalmos. Some of the results of these assays are shown in Table 1. See Figure 1.

With the above assay data available, an analysis of the various chemical procedures used in the preparation of these pituitary fractions pointed the way to a method for specifically separating the TSH and the EPS in appropriate pituitary extracts. Since thyrotropin preparation No. 11

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[2] This method of assay differs from that of Albert (1945) in two ways. Albert classified the exophthalmic response as present or not present in each fish when grossly compared to controls. The value for each group of fish was recorded as the percentage of positive responses.

TABLE 1. THE ASSAY OF EXOPHTHALMOS PRODUCING SUBSTANCE (EPS) IN A VARIETY OF PITUITARY FRACTIONS[1]

Unknown preparation	Constituents[2] known to be present	Av. % increase intercorneal distance		
		Unknown	KD44A (standard)	Saline control
1	TSH 83%	14.3±2.7	16.4±1.5	0.5±1.0
		15.1±2.3	15.5±2.3	0.5±1.7
2	TSH 38%	5.3±1.8	16.4±1.5	0.5±1.0
3	TSH 35%	2.5±1.0	16.4±1.5	0.5± .0
4	TSH 28%	2.4±1.1	19.0±3.3	2.8±2.4
5	TSH 45%	8.3±0.9	19.0±3.3	2.8±2.4
6	TSH 55%	15.0±3.0	16.1±2.8	2.8±1.8
7	TSH 10%; LH high; FSH low	19.9±2.7	16.1±2.8	2.8±1.8
		19.2±0.59	15.6±2.0	−0.9±0.6
8	TSH low; LH low; Prolactin high	3.0±0.8	14.3±2.3	2.6±0.7
		4.8±1.5	11.8±1.7	2.5±0.2
9	TSH 150–200%; LH low; Prolactin low	6.2±1.2	11.8±1.7	2.5±0.2
10	TSH 30%; LH low; FSH high	3.8±1.2	14.1±1.9	−0.7±0.8
11	TSH 1000%; LH low	2.2±2.6	13.1±2.6	1.2±1.0
12	TSH 10%; LH high	10.5±0.6	5.3±2.7	0.5±1.1
13	ACTH moderate	0.1±1.6	12.5±1.8	—
14	TSH 100%; LH low; Growth low; ACTH low	19.4±2.1	17.8±2.1	0.5±1.4
15	TSH 300%; LH low; Growth low; FSH low	18.4±2.4	16.5±2.7	−2.1±1.5
16	Growth high	0.2±2.0	16.4±1.5	0.5±1.0
17	TSH 100%; LH very high	17.7±2.5	16.8±1.9	0.6±0.47
18	FSH high; TSH <10%; ACTH 0.0096 USP μ/mg.; Growth nil	0.5±0.56	10.0±1.36	1.0±0.85
19	TSH very low; LH high; FSH high	7.4±3.17	14.8±4.44	0.6±1.81

It will be noted that in Experiments #7, #11, #12, and #19, the known potency of TSH did not coincide with the exophthalmos producing activity demonstrated.

[1] 0.25 mg. of powder used per fish.

[2] TSH = Thyroid Stimulating Hormone potency, recorded as per cent of Armour standard 2R3 (see #14) which is regarded as 100%; LH = Luteinizing Hormone; FSH = Follicle Stimulating Hormone; ACTH = Adrenocorticotropic Hormone; and Growth = Growth Hormone.

FIG. 1. An example of the exophthalmic response produced in Fundulus by the administration of an anterior pituitary extract rich in the exophthalmos producing substance (EPS). The intercorneal distance of the fish on the left increased 13.2% in 6 hours. The fish on the right served as a control.

TABLE 2. ASSAYS OF EXOPHTHALMOS PRODUCING SUBSTANCE (EPS) IN THE RESIDUE (R) AND SOLUBLE (S) FRACTIONS PREPARED FROM PITUITARY EXTRACTS KNOWN TO HAVE A CAPACITY TO STIMULATE THE THYROID AND TO PRODUCE EXOPHTHALMOS

Preparation	Average percentage increase in intercorneal distance[1]			
	Unknown		KD44A (standard)	Saline (control)
	0.25 mg.	0.125 mg.	0.25 mg.	
15[2]	14.5±2.1 (18.4±2.4)	10.5±1.8 (12.4±1.7)	11.8±1.7	0.59±1.6
183S	0.3±0.55	−0.7±1.0	11.7±1.7	0.59±1.6
183R	13.0±1.52	7.2±1.07	11.7±1.7	0.59±1.6
IRW[2]				
$224S_1$	−1.4±0.71		9.7±1.82	0.14±0.82
$224S_2$	−0.9±0.79		9.7±1.82	0.14±0.82
$224R_2$	7.6±1.7		9.7±1.82	0.14±0.82

Compare the results of the EPS assays on the residue (R) and soluble (S) fractions appearing in this table with the results of the TSH assays on the same fractions appearing in Table 3. EPS is contained in the R fractions.

[1] All percentages are expressed as a positive increase unless specified by a minus sign. Maximal increase in intercorneal distance usually occurred about 6 hours following injection. Therefore, the data acquired in 6 hours have been recorded. In instances where further increase was observed at 17 hours, the values have been recorded in parentheses.

[2] Starting material (same preparation as No. 15, Table 1).

(Table 1) contained little if any exophthalmos producing activity, yet the material from which it was derived was rich in it; and since it represented the soluble fraction from trichloracetic acid precipitation, this method was applied to a new starting material, pituitary extract No. 15, which had been found to produce both exophthalmos and thyroid hyperplasia.

Beef thyrotropin (No. 15 in Tables 1 and 2) was dissolved in distilled water to make a 1% solution. Trichloracetic acid[3] was added to make a concentration of approximately 8%. After thorough stirring, the mixture was allowed to stand at 3–5°C. for 1 hour. Following centrifugation the precipitate was separated from the supernatant, taken up in water (one-half of the original volume), brought to neutrality by the addition of sodium carbonate, dialyzed until free of salt, and lyophilized. The supernatant solution, that had resulted from the above trichloracetic acid precipitation, was also neutralized, dialyzed, and lyophilized. The activities of EPS and TSH in the residue (183R) and in the supernatant (183S) were assayed. The EPS was entirely confined to the residue fraction (183R) and the TSH was predominantly in the soluble fraction (183S). The results of the assay for EPS in each fraction are shown in Table 2. The results of several methods of assay for TSH in these fractions are shown in Table 3.

[3] Trichloracetic acid precipitation has been used by some authors (Loeser, 1932; Ciereszko, 1945; Albert, 1949) as a step in the purification of thyroid-stimulating activity of pituitary extracts; however, in their purification procedure the TSH was recovered in the supernatant fluid and the precipitate served to remove materials other than TSH.

TABLE 3. ASSAYS FOR TSH IN FRACTIONS LISTED IN TABLE 2

	Total dose/chick (gamma)	Av. chick thy. wt.[1]	Control thy. wt.[1]	Mean cell ht. (microns)[2]	Iodine depletion[3]
				Control (2.89 ± .040)	
15[4]	50	4.19 ± .44	3.53 ± .22		
	100	6.35 ± .49	3.53 ± .22		1.2–1.3 u/mg.
	200	6.85 ± .48	3.53 ± .22		
	400	7.13 ± .45	3.98 ± .15	5.38 ± .038	
183S	50	5.35 ± .30	3.53 ± .22		
	100	5.72 ± .40	3.53 ± .22		3.5 u/mg.
	200	5.11 ± .44	3.53 ± .22		
	400	6.88 ± .53	3.98 ± .15	5.02 ± .02	
183R	50	4.35 ± .29	3.53 ± .22		
	100	4.82 ± .41	3.53 ± .22		0.6 ± 0.8 u/mg.
	200	5.81 ± .28	3.53 ± .22		
	400	5.50 ± .36	3.98 ± .15	3.79 ± .036	
IRW[5]				Control (2.73 ± .034)	
$224S_1$	100	6.49 ± .28	3.97 ± .18	5.25 ± .034	2.0 μ/mg.
$224S_2$	100	8.26 ± .45	3.97 ± .18	5.84 ± .063	3–4 μ/mg.
$224R_2$	100	4.78 ± .16	3.97 ± .18	3.40 ± .033	0.25 μ/mg.

Compare the results of the TSH assays on the residue (R) and soluble (S) fractions appearing in this table with the results of the EPS assays on the same fractions appearing in Table 2. Most of the TSH is in the S fraction. It was shown in Table 2 that EPS was confined to the R fraction.

[1] Based on 9 to 11 chicks per group.

[2] Based on 7 to 10 thyroids in each group. A representative cell in each of 50 acini was measured in each gland.

[3] USP units.

[4] Starting material (same preparation as No. 15, Table 1).

[5] Starting material.

Although TSH was thus obtained free from EPS, the EPS fraction contained some TSH. In order to confirm the above observations and to improve the purification of the EPS, another new starting material (pituitary extract, No. IRW) was treated in the same manner as described above except that the precipitate produced by trichloracetic acid (residue fraction No. $224R_1$) was suspended in enough water to effect solution and reprecipitated with trichloracetic acetic acid as above. The double precipitation procedure thus yielded two soluble fractions (1st, No. $224S_1$; 2nd, No. $224S_2$) and one residue fraction ($224R_2$). In Tables 2 and 3, it may be seen that the two soluble fractions ($224S_1$ and $224S_2$) contained TSH free of EPS, and that the reprecipitated residue fraction ($224R_2$) contained EPS which was relatively free from TSH. It is also evident that considerable TSH was removed from the first residue fraction by the re-precipitation procedure.

DISCUSSION

The experimental evidence which has been presented indicates that when anterior pituitary extracts are administered to animals, TSH *per se* is not responsible for the development of exophthalmos. It appears that there is another substance which produces the exophthalmos. We have proposed to call this exophthalmos producing substance (EPS).

These observations give support to the suspicion registered by one of the authors (Dobyns, 1946a, 1946b) and Jefferies (1949) that TSH might not be the substance which produces exophthalmos. Since known as well as unknown factors still remain in most anterior pituitary preparations, it is possible that a substance which inhibits exophthalmos may be removed from some preparations while being concentrated in others. This question is raised because thus far the EPS preparations still appear to have some TSH impurities in them. The possibility of an inhibitory substance being responsible for these findings seems very unlikely since it is possible to eliminate EPS from TSH and to eliminate TSH almost entirely from EPS (see Tables 2, 3; 224 series).

The theory that TSH *per se* is responsible for clinical exophthalmos has not been consistently supported by the results of assays in clinical subjects (Starr and Rawson, 1937; Purves and Griesbach, 1949). The failure to show a high titre of TSH in all patients suffering from progressive exophthalmos has perhaps been more frequently attributed to the inadequacy of the assay methods than to the possibility that TSH might not be responsible for the problem concerning the eyes. As a result of the findings described here, an assay method for the exophthalmos producing substance in selected clinical subjects is now being explored.

SUMMARY

It has been found in a variety of pituitary extracts studied that the thyroid stimulating hormone (TSH) and the exophthalmos producing substance (EPS) in anterior pituitary extracts do not always parallel one another. An analysis of the various chemical procedures by which these preparations were obtained lead to the separation of TSH from EPS. These two substances were separated by their differential solubility in trichloracetic acid. EPS was assayed in Fundulus and TSH was assayed in day-old chicks. The results indicate that EPS and TSH are not the same substance.

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RESPONSE OF INBRED LINES OF CHICKENS TO THIOURACIL[1]

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IT HAS long been known that there are wide species, sex and age differences in thyroid activity. It is also known that environmental factors such as season and temperature, iodine intake, pituitary activity, administration of goitrogens and the alteration of endocrine activity by other means may have a profound effect on thyroid function. In recent years evidence has been accumulating with respect to the influence of genetic makeup or constitution on thyroid function within species.

During the past half century poultry breeders have greatly improved the type, productivity and efficiency of the various breeds of chickens such as the White Leghorn, Barred Plymouth Rock, Rhode Island Red, New Hampshire and others. In addition, they have developed strains or lines of these breeds which are especially noted for such characteristics as egg number, egg size, rate of growth, rate of feathering, and so on. With such changes in phenotype and genotype, it is only logical to attempt to correlate these differences with specific physiological characters.

Several investigators have reported breed and strain differences in thyroid activity of chickens. Schultze and Turner (1945) found that thyroxine secretion rate on a body weight basis was greater in White Leghorn males than in White Plymouth Rock males. Mixner and Upp (1947) compared single cross hybrid parents with their 4-way cross hybrid progeny, and found increased thyroxine secretion rate in the hybrid offspring. They suggested that increased thyroxine secretion rate might be a factor in heterosis or hybrid vigor. Glazener, Shaffner and Jull (1949) found that thyroxine secretion rate, measured only in female chicks, was higher in rapid-growing strains. Thyroprotein depressed growth in slow-growing New Hampshires and rapid-growing Barred Rocks. It was concluded that "each strain had a physiological optimum level of thyroid activity for growth, feed utilization and feathering." El-Ibiary and Shaffner (1950) selected chicks for large and small thyroid size; selection was on a family

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basis from birds fed a normal ration. The chickens from lines selected for large and small thyroid size were then fed thiouracil and it was possible to develop lines which differed in responsiveness to thiouracil. Boone *et al.* (1950) measured thyroxine secretion rate in fast- and slow-feathering Rhode Island Reds. Thyroprotein administration increased rate of feathering in the slow-feathering strains. The daily d,l-thyroxine secretion rate was slightly higher in the slow-feathering birds, although correction for body weight showed thyroxine secretion to be somewhat higher per unit of body weight in the fast-feathering chickens. These authors concluded that the evidence available does not indicate that slow-feathering is due to an inherently low thyroid secretion rate.

The experiments reported in this study were carried out to compare various inbred lines of chickens in response to thiouracil.

PROCEDURE

Five distinct lines of Leghorn chickens, obtained from a commercial source, were used.

Two separate pens of parent stock of line 1 were maintained. The chicks from these pens were designated as lines 1A and 1B and were supposedly of identical genetic make-up. The amount of inbreeding of each was, therefore, the same—37½%. Line 2 had been inbred for seven generations and had an inbreeding coefficient of 78%. Line 3 also had an inbreeding coefficient of 78%, and line 4 an inbreeding coefficient of 59%. Line 5 was non-inbred. The 5 lines were not of common origin.

Trials I and II were replications in successive weeks. In trial III a comparison was made of the variability within the highly inbred line 2 and line 1A which had an inbreeding coefficient approximately half as great (37½%). In trial IV comparisons were made of crosses of 3 of the lines studied in the first 3 trials. It was anticipated that the 3 crosses would be similar; two crosses were reciprocal matings of lines 1 and 2. There was also a cross of line 1 with line 3. Line 3 was similar to line 2 in 13-day gain, thyroid weight and relative thyroid weight, as may be seen in Table 1. Thus it was expected that the cross of lines 1 and 3 might be similar to the crosses of lines 1 and 2.

The chicks were received on the second day after hatching. They had been sexed and were males, with the exception of a negligible number of females. Half the chicks of each group were fed a standard chick starting ration and the remainder received the same ration to which thiouracil had been added at a level of 0.2%. The initial weight of each chick was taken. At the end of 13 days the chicks were again weighed and were then killed and the thyroid glands removed and weighed.

The data analyzed were: 13-day gain in grams, thyroid weight in milli-

grams, and relative thyroid weight (thyroid weight as a percentage of body weight).

The gains and the thyroid weights were analyzed as grams and milligrams, respectively, and also as logarithms of those values. The object of making both analyses was to learn whether certain effects tended to be constant in absolute units or percentage-wise. No clear-cut conclusion is possible regarding the form in which the data have homogeneous variance,

TABLE 1. EFFECT OF THIOURACIL ON THYROID WEIGHT AND RATE OF GAIN IN TRIALS I AND II[1]

Line	Inbreeding coefficient	Thiouracil treatment	Thyroid weight, mg.	Thyroid wt. as % of body wt.	13-day gain, gm.
1 A	37.5%	None	9.8	0.0089	75.2
		0.2%	26.1	.0295	52.5
1 B	37.5%	None	10.3	.0096	71.6
		0.2%	24.0	.0290	49.8
2	78.0%	None	8.2	.0099	50.0
		0.2%	13.1	.0192	35.8
3	78.0%	None	7.5	.0093	51.1
		0.2%	14.9	.0223	34.9
4	59.0%	None	9.8	.0095	67.0
		0.2%	18.5	.0217	49.1
5	0	None	8.8	.0074	84.8
		0.2%	25.5	.0268	58.3

[1] The number of chicks of a line on a diet varied from 21 to 25.

TABLE 2. A COMPARISON OF TWO INBRED LINES IN RESPONSE TO THIOURACIL, TRIAL III

Line	No. of chicks	Inbreeding coefficient	Thiouracil treatment	Thyroid weight, mg.	Thyroid wt. as % of body wt.	13-day, gain gm.
1 A	22	37.5%	None	13.7	.0124	78.9
	43		0.2%	33.6	.0359	58.9
2	21	78.0%	None	9.1	.0102	61.6
	38		0.2%	18.7	.0272	38.5

since the evidence regarding homogeneity is conflicting in the various trials with the data in either form. However, the same conclusions were reached regarding the significance of line and diet effects regardless of the form in which the data were analyzed.

RESULTS AND DISCUSSION

The data for all 4 trials are summarized in Tables 1, 2, 3 and 4.

Thyroid weight

As expected, thiouracil produced a highly significant ($P < 0.01$) increase in thyroid weight in all 4 trials. In each of the 3 trials of inbred lines, the

lines differed highly significantly in thyroid weight. Also as expected, the 3 crosses in trial IV had similar thyroid weights: These weights were very similar for the reciprocal crosses. The weight differences were also small between the reciprocal crosses and the third cross. All of these differences were so small that they were far from significant.

In each of the 4 trials, the increase in thyroid weight due to thiouracil varied from inbred line to inbred line or from cross to cross. (In other words, there was diet and line (or cross) interaction.) These variations (interactions) were highly significant whether the analysis was in milligrams or logs. In the 4 trials, the weight of the thyroid with thiouracil was 194% to 241% of the weight without.

TABLE 3. EFFECT OF THIOURACIL ON LINE CROSSES, TRIAL IV[1]

Line cross	Thiouracil treatment	Thyroid weight, mg.	Thyroid wt. as % of body wt.	13-day gain gm.
1B×2	None	11.0	.0106	73.0
	0.2%	25.3	.0288	56.0
2×1A	None	11.0	.0102	74.1
	0.2%	24.0	.0265	54.7
1A×3	None	9.6	.0097	72.5
	0.2%	29.7	.0342	57.5

[1] The number of chicks of a cross on a diet was 12 or 13.

TABLE 4. VARIATION OF CHICKS OF TWO INBRED LINES WITH DIFFERENT INBREEDING COEFFICIENTS, TRIAL III

Line	Inbreeding coefficient	Thiouracil treatment	Standard deviation	Coefficient of variation
		13-day gain		
1 A	37.5%	None	8.9 gm.	11%
2	78%	None	7.9 gm.	13%
1 A	37.5%	0.2%	5.9 gm.	10%
2	78%	0.2%	5.6 gm.	15%
		Thyroid weight		
1 A	37.5%	None	4.1 mg.	29%
2	78%	None	3.8 mg.	41%
1 A	37.5%	0.2%	8.8 mg.*	26%
2	78%	0.2%	4.8 mg.*	26%

* These 2 standard deviations differ significantly ($P < 0.2\%$). The members within the other 3 pairs of standard deviations are not significantly different.

For thyroid weight, the 5 lines performed similarly with respect to each other in the various trials. There was moderate—but nonsignificant—evidence of diet and trial interaction for thyroid weight; the increase due to thiouracil was least in trial II and most in III.

Relative thyroid weight

No tests of significance were made of effects on relative thyroid weight, but some conclusions appear justified. Relative thyroid weight was in-

creased greatly by thiouracil in all 4 trials and with all lines and crosses. However, the effect of thiouracil varied considerably among lines and among crosses. In trials I and II, the relative thyroid weight was increased least in line 2, more in lines 3 and 4, and most in line 5 and in the 2 groups of line 1. In trial III also, the effect was considerably less in line 2 than in 1. In trial IV the effect was greater in the cross of lines 1 and 3 than in the reciprocal crosses of lines 1 and 2.

13-day gain

In the selection or development of strains or lines of various domestic animals, growth rate is of great economic importance. As expected, the induction of hypothyroidism with thiouracil had a highly significant depressing effect on rate of gain in all lines and crosses in all 4 trials. The lines (in trials I, II and III) differed highly significantly in gain. See Tables 1 and 2. Line 5 gained most rapidly; line 1 was second in gain, and lines 2 and 3 gained least. On the control diet, line 5 gained 70 % more than line 2, and the average gain of the 2 groups of line 1 was 56 % greater than the gain of line 2. As anticipated, the crosses in trial IV differed little in gain.

When gains in grams were analyzed, it was found that the depression from thiouracil was significantly different for the various lines, i.e., there was significant diet and line interaction. However, the evidence of interaction was negligible for logs. This means that the depression was a nearly constant percentage of the gain without thiouracil. In each trial the depression was close to 30 %. From trial to trial, also, the depression in gain due to thiouracil was 30 %, but the depression in grams was different for trials in which the gain on the control diet differed.

Relation of gain and thyroid weight

Coefficients of the relation of gain and thyroid weight were not calculated for chicks of the same inbred line because dot diagrams indicated no appreciable relation. However, correlation coefficients were computed using inbred line averages. One of the variables correlated was the average gain in grams on both diets; the other was the average thyroid weight in milligrams on the thiouracil diet. The correlation coefficient was 0.92.

The comparative variation of line 1 (inbreeding coefficient 37.5%) and line 2 (inbreeding coefficient 78%) was studied in trial III. Only for these 2 lines and only in trial III was it possible to calculate the variation of chicks with the same sire and dam. Table 4 shows standard deviations and coefficients of variation. In all 4 cases (i.e., 13-day gain and thyroid weight on each of the 2 diets) line 1A varied more than line 2, although the difference in the standard deviations was statistically significant (with $P < 0.2\%$) only for thyroid weight on the thiouracil diet.

There were too few sires and dams to make worthwhile estimating the components of chick variance due to sires and dams.

Comparison of lines and their crosses

It seems reasonable to compare lines 1 and 2 in trial III with their reciprocal crosses in trial IV, since these trials were largely concurrent. (Ten of 13 days were common to the 2 trials.) On each diet, the gain of the reciprocal crosses was between the gains of the 2 parental lines, but was closer to the gain of the faster-gaining line, 1. This suggests some hybrid vigor. The respective average gains in grams for line 1, the reciprocal crosses, and line 2 were: 79, 74, and 62 on the control diet, and 59, 55, and 39 with thiouracil.

Thyroid weights of the reciprocal crosses were more nearly midway between the weights for the parents. The average thyroid weights in milligrams for line 1, the crosses, and line 2 were: 14, 11, and 9 on the control diet, and 34, 25, and 19 with thiouracil.

Relative thyroid weights of the crosses were similar to those of the parent with the lower relative weight. The values in percentage in the same order as previously given were: 0.0124, 0.0104, and 0.0102 with the control diet, and 0.0359, 0.0276, and 0.0272 with thiouracil.

These studies provide additional evidence of endocrine differences between lines or strains of the same species. They are similar in general nature to the conclusions of Boettiger (1941), Koger *et al.* (1942), Hurst and Turner (1948) and Stob and Andrews (1952) that strains of mice differ in thyroid physiology. They likewise support the conclusions of Mixner and Upp (1947), Glazener *et al.* (1949), El-Ibiary and Shaffner (1950) and Boone *et al.* (1950) that strains or lines of chickens vary widely in thyroid function.

In any breeding program it is desirable to determine the characteristics of the progeny at the earliest possible time and during the shortest practical period of observation. If strain differences in thyroid function are important, and there is every reason to believe thay are, it seems obvious that they can be detected in the young chick (14 days of age) and without elaborate procedures. Highly significant differences in thyroid weight and in 13-day gain were readily detected. An extremely high correlation of rate of gain and thyroid weight on the thiouracil diet was found ($r = 0.92$).

Since one of the basic principles of selection and breeding is the reduction in variability, it was of interest to measure the effects of inbreeding upon such specific factors as thyroid size and response to thiouracil. In general there was less variation in a line with an inbreeding coefficient of 78% as compared with one of $37\frac{1}{2}\%$.

From the genetic standpoint, the comparisons of lines and their crosses is of special interest. Gross thyroid weight of the reciprocal crosses was about midway between the weights of the parents. When thyroid weights was expressed as a percentage of body weight, the relative thyroid weights of the crosses were similar to those of the parent with the lower relative gland weight.

SUMMARY

Five lines of chickens, varying in inbreeding coefficients from 0 to 78%, were fed thiouracil at a level of 0.2% of the diet from the second to the 14th days post-hatching. There were considerable differences between lines in gross and relative thyroid weight. Growth rate, expressed as 13-day gain, varied highly significantly between lines. Thiouracil had a highly significant depressing effect on rate of gain. Thiouracil depressed the growth rate about 30%. Thus the rate was depressed more *in grams* in a fast-growing line than in one which grew slowly. Using inbred line averages, the correlation coefficient of 0.92 was obtained when 1 variable was the average gain in grams on both diets and the other variable was the average thyroid weight in milligrams on the thiouracil diet.

Thyroid weight and 13-day gain of chicks with the same sire and dam varied more in line 1 (37.5% inbred) than in line 2 (78% inbred).

Line 1 and 2 and their reciprocal crosses were compared. The gains of the reciprocal crosses were between the gains of the two parental lines but closer to the gains of the faster-gaining line, 1. Gross thyroid weights of the crosses were nearly midway between the gland weights of the parents. Relative thyroid weights of the crosses were similar to those of the parent with the lower relative thyroid weight.

These studies provide additional evidence of endocrine differences between lines or strains of the same species.

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NOTES AND COMMENTS

SURVIVAL OF MATING BEHAVIOR IN MALE RATS AFTER THYROID-PARATHYROIDECTOMY[1]

Young, Rayner, Peterson and Brown (1952) have recently reported that thyroidectomy does not alter the copulatory behavior of male guinea pigs. Their study included objective and quantitative measures of coital activity before and after operation. No comparable evidence is available with respect to the male rat and such casual observations as have been reported are contradictory, some writers stating that loss of the thyroid eliminates or greatly reduces sexual responses (Rickey, 1925) and others asserting that the behavior is unchanged (Hammett, 1922). The present investigation was conducted to measure pre- and postoperative behavior in standardized fashion.

SUBJECTS AND METHODS

Thirteen adult male albino rats 90 to 150 days old at the start of the experiment were given a series of preoperative mating tests with receptive females. The method of testing for sexual vigor has been described in earlier publications and has been proven to be highly reliable (Beach, 1944). The most important responses recorded are incomplete copulations, intromissions, and ejaculations. Each animal was tested until he had copulated (but not necessarily ejaculated) in a minimum of 3 tests. The thyroid and parathyroid glands were removed, 0.5% calcium gluconate was added to the stock diet, and 8 postoperative mating tests were conducted in the course of the next 30 days. For 30 days after the final test 0.2% thiouracil was added to the diet in the expectation that if the operation had been incomplete any residual glandular tissue would hypertrophy and be more easily discovered in the postmortem examination. When the neck region was examined under low magnification small tabs of tissue which might have been thyroid were discovered in only 3 rats, and in each case these fragments were sectioned and stained and proved to be nonglandular.

RESULTS AND CONCLUSIONS

Comparison of mating performance in pre- and postoperative tests revealed no significant differences. Three males which had not ejaculated before operation did so afterwards and one case failed to ejaculate postoperatively although this response had occurred previously. The average frequency of intromissions was 7.0 per test before and 7.4 after operation, a difference which was not statistically significant. The frequency of incomplete copulations did not change significantly.

These findings are in harmony with the thesis that secretions of the thyroid and parathyroid glands are not essential to normal mating behavior in the male rat.

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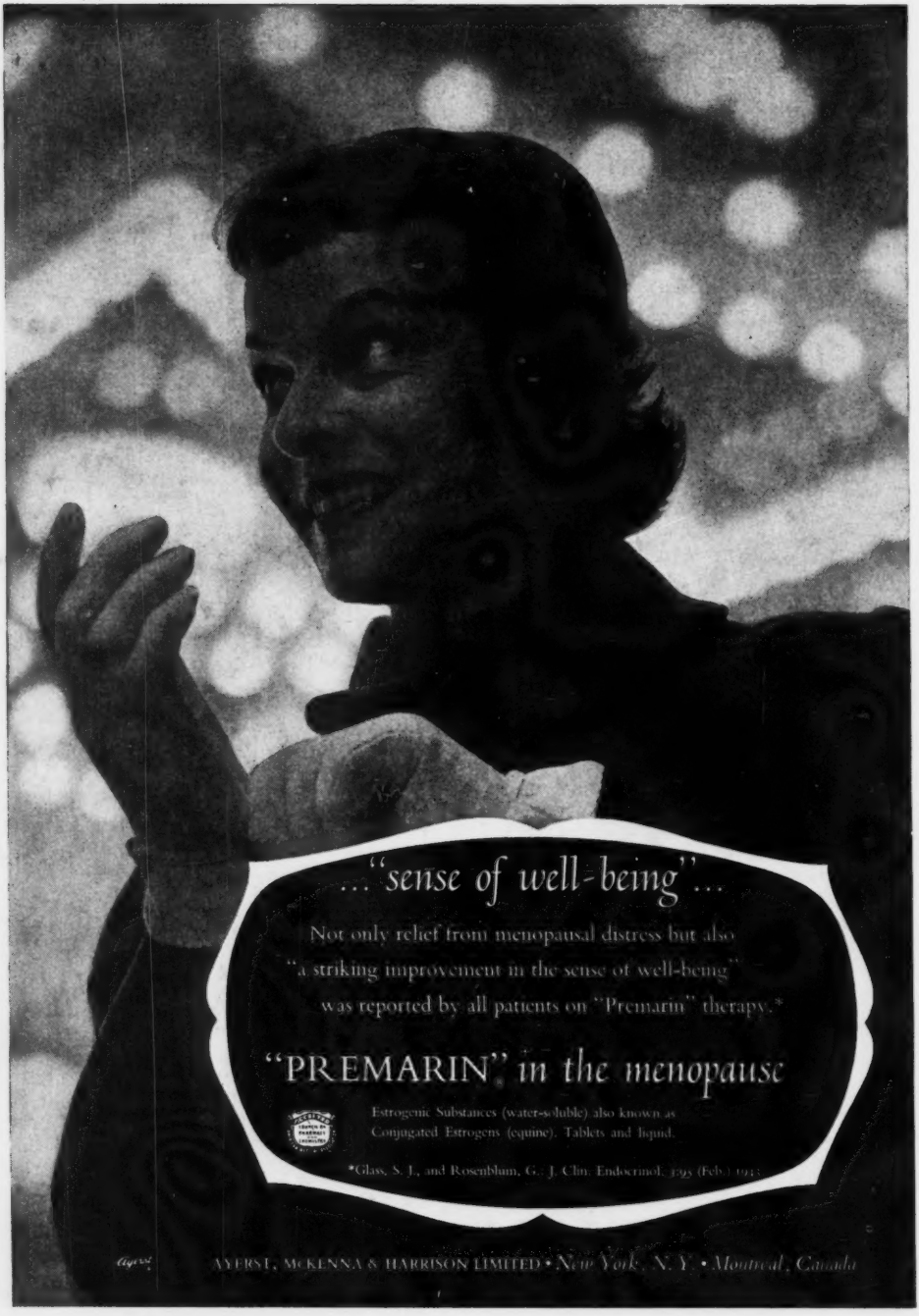
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